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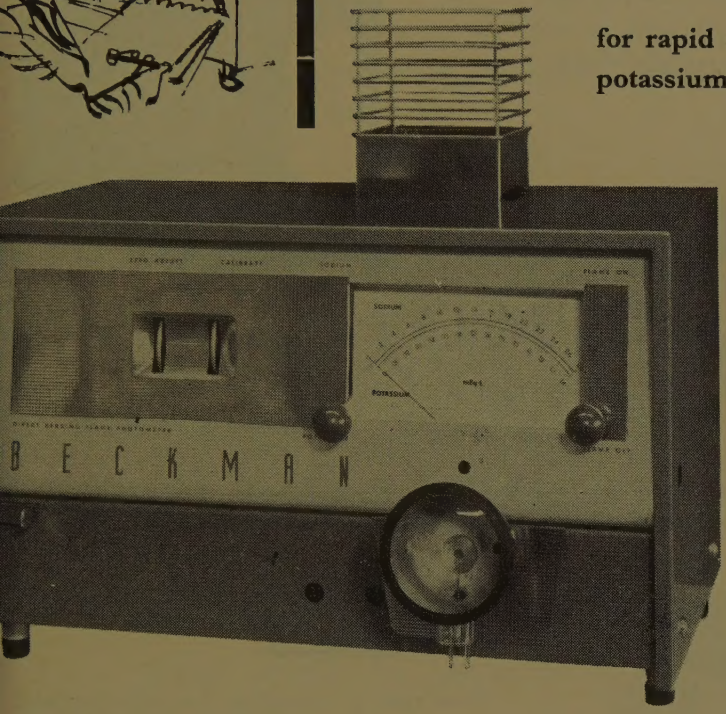

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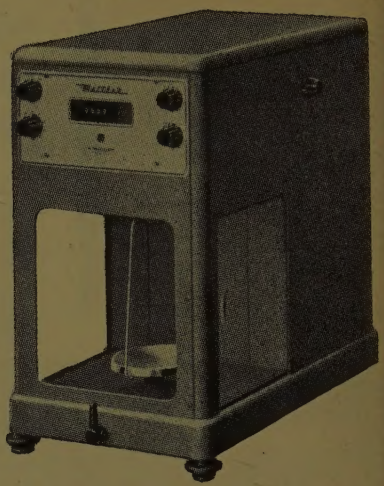

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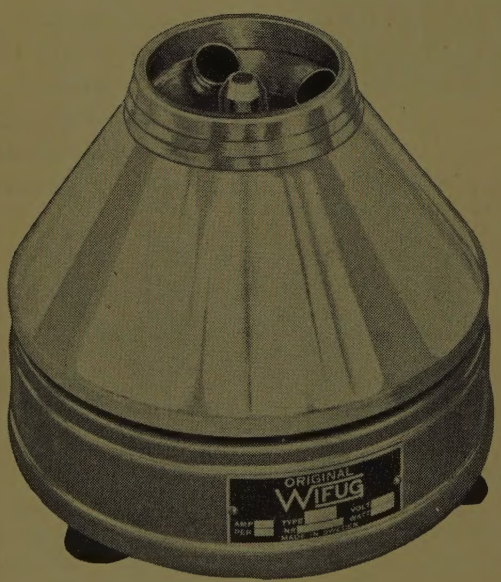

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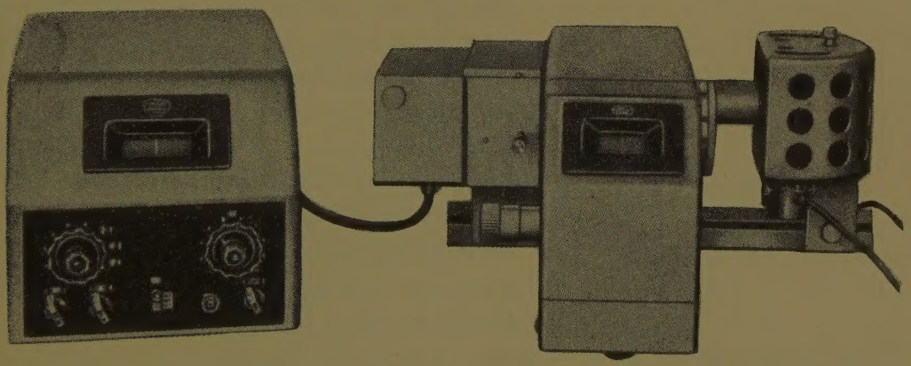

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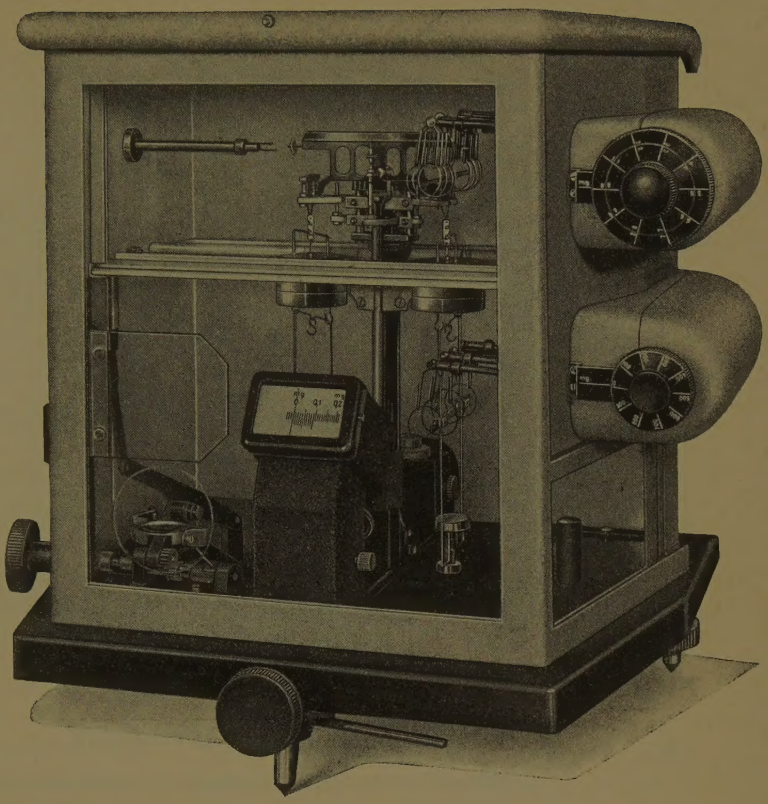

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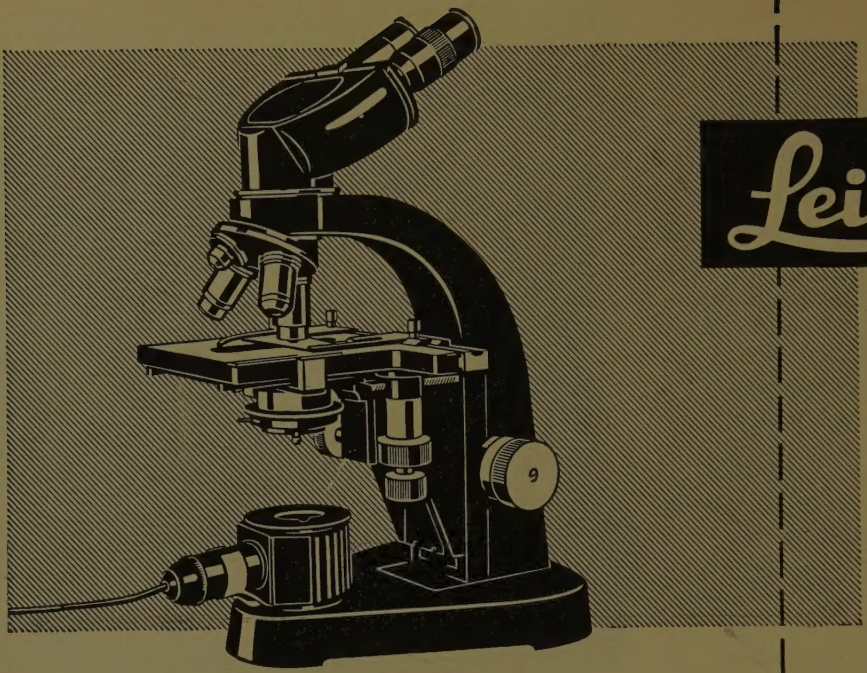

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HE INFLUENCE OF METHEMOGLOBIN AND STORAGE UPON THE SPECTROPHOTOMETRIC DETERMINATION OF CARBON MONOXIDE IN BLOOD

By M. HARBOE

From The Institute of Aviation Medicine, Royal Norwegian Air Force, Oslo, Norway

(Received for publication May 16, 1957)

In our laboratory, we have been in need a method for the determination of carbon noxide in capillary blood; a reliable me- d which could be used on blood samples n after a few days of storage. The micro- ometric method of Scholander and Rough- (1943) requires considerable technical ll, and we would, therefore, prefer a simp- spectrophotometric procedure for routine rk.

The spectrophotometric determination of bonylhemoglobin (HbCO) in blood has n described by Hüfner (1900) and Heil- yer (1933). In their methods the optical sity is determined at 576, 560 and 541 mμ he 2-pigment system HbO_2-HbCO. The os D 541/D 560 and D 576/D 560 are culated, and the percentage of HbCO is read from a calibration curve. Hartmann 37), Steinmann (1939) and Drabkin 50) advocate the use of similar or slightly dified methods.

Klendshoj, Feldstein & Sprague (1950) recommend the use of $Na_2S_2O_4$ to reduce HbO_2 to Hb. The optical density is read at 555 and 480 mμ in the 2-pigment system Hb-HbCO. The ratio D 555/D 480 is calculated and the percentage of HbCO is read from a calibration curve. In our hands, the addition of $Na_2S_2O_4$ leads to an unstable system with continously changing optical density at the two wavelengths with corresponding change in the ratio. The method, therefore, requires very accurate timing in the readings and we have preferred to use the method described by Heilmeyer.

In this work, the results obtained by the spectrophotometric determination are presented as compared to those obtained by a gasometric technique (Scholander & Roughton, 1943). Knowing that methemoglobin (MHb) is a normal constituent of blood (Paul & Kemp, 1944), and that MHb may be formed when blood is allowed to stand,

Table I. *The spectrophotometric constants used for calculation of carbonylhemoglobin percentages in blood; determined by measuring the optical density at 576,560 and 541 mμ in 12 different blood samples.*

Spectrophotometric ratio	Mean	S. D.
$\frac{D\ HB\ O_2\ 576}{D\ HB\ O_2\ 560}$	1.783	0.005
$\frac{D\ HB\ CO\ 576}{D\ HB\ CO\ 560}$	0.885	0.009
$\frac{D\ HB\ O_2\ 576}{D\ HB\ CO\ 560}$	1.268	0.009
$\frac{D\ HB\ O_2\ 560}{D\ HB\ CO\ 560}$	0.711	0.004
$\frac{D\ HB\ O_2\ 541}{D\ HB\ O_2\ 560}$	1.672	0.004
$\frac{D\ HB\ CO\ 541}{D\ HB\ CO\ 560}$	1.135	0.005
$\frac{D\ HB\ O_2\ 541}{D\ HB\ CO\ 560}$	1.189	0.009

the influence of MHb on the results has been studied. Finally, data are presented on the influence of storage upon the determination.

METHODS

Capillary blood, which is obtained from the ear or a finger tip, is stored in glass capillary tubes according to a technique recommended to us by B. Fallentin. The capillary tubes, prepared with sodium citrate to prevent coagulation and closed with rubber plugs, contain 20—30 μl blood. The contents are expelled directly into 5 ml. 0.4 per cent NH_3 for subsequent analysis as varying concentration of this solution within wide limits does not interfere with the results. The solution is placed in a standard cuvette, and the optical density is read at 576, 560 and 541 mμ. We have used a Beckman spectrophotometer model DU, slit 0.02 mm, temperature 20° C. The ratios D 576/D 560 and D 541/D 560 are calculated and the percentage of HbCO is then read from the calibration curves. The values are presented as the mean values of three parall determinations. The calibration curves have bee constructed according to the formulas given b Drabkin (1950). The spectrophotometric constant used (Table I) have been found by analysis HbCO-free blood and blood saturated with CO For HbCO-free blood, citrated venous blood fro a non-smoker was equilibrated with oxygen fo 30 minutes and then dissolved in NH_3 for analysi For blood saturated with CO, CO was bubble through this solution for 30 minutes prior to ana lysis. Sufficient amounts of CO were then kept i physical solution in the NH_3 to secure saturatio of the Hb with CO during the analysis.

MHb has been determined by the method Evelyn & Malloy (1938). 2.5 ml blood is dissolve in 10 ml phosphate buffer to secure a high degre of accuracy at small amounts of MHb. The result are presented as the mean value of 2 parall determinations.

RESULTS

Table II shows the results obtained by th spectrophotometric determination as compa red to those obtained by Scholander Roughton's microgasometric method. Wit up to 30 per cent HbCO there is good agree ment between the two methods. The spectro photometric values are lower than the gaso metric values at high percentages of HbC as part of the CO may then escape durin the analytical procedures. To get an estima of the reproducibility of the results, 8 ana lyses were then performed on the sam sample of blood. A range of 5.8—7.0 per ce HbCO was found with a mean value of 6 per cent for both wave length combination

The spectrophotometric determination hemoglobin derivatives is influenced by tem perature (Maxfield, Bazett & Chamber 1941; Refsum 1957). We have been able confirm this finding as regards the dete mination of HbCO, and have found that

ble II. *Carbonylhemoglobin in per cent tal hemoglobin as determined by spectro-otometric and gasometric method in 17 blood samples.*

ctrophotometric method		Gasometric method
76/D 560	D 541/D 560	
1.0	2.0	2.0
3.0	2.0	2.0
3.0	3.0	2.5
4.0	4.0	4.0
5.0	6.0	5.5
7.0	6.5	6.5
7.0	7.5	8.0
10.5	11.5	11.0
12.0	12.0	12.0
14.5	15.0	15.5
18.0	20.0	19.0
24.0	25.0	22.5
28.5	29.5	31.0
66.5	68.0	77.0
84.0	86.5	96.0
90.5	90.0	100.9
91.0	93.0	99.5

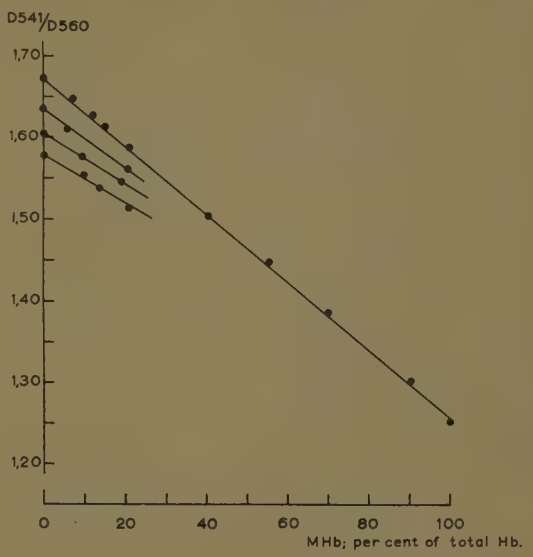

Fig. 1. The ratio D 541/D 560 obtained by analysis of four blood samples with different concentration of carbonylhemoglobin at increasing concentration of methemoglobin.

tant temperature during analysis is of importance for the accuracy of the nod.

Influence of methemoglobin.

ne influence of MHb upon the spectro-ometric determination of HbCO has investigated for an admixture of app-mately 0—20 per cent MHb, as only l amounts of MHb are apt to be formed ng storage. MHb was added in increasing unts to blood samples which contained 0, and 12.5 per cent HbCO respectively. ratios D 576/D 560 and D 541/D 560 MHb percentages were determined; the s being corrected for the slight decrease er cent HbCO of total Hb, as total Hb creased when MHb is added to the blood ple. Figure 1 shows the straight-line decrease of the ratio D 541/D 560 at increasing amounts of MHb. Similar observations have been made as regards the ratio D 576/D 560. Michel & Harris (1940) have described a similar straight-line decrease of the ratio D 576/D 560 for HbCO-free blood with increasing amounts of cyanmethemoglobin. If MHb is found in a blood sample containing a certain amount of HbCO, the ratio D 541/D 560 which would have been found by analysis of the same sample with a minimal amount of MHb can be deduced from Figure 1. Accordingly, the HbCO percentage of a blood sample can be determined even when MHb has been formed during storage.

Influence of storage.

Figure 2 shows the ratio D 541/D 560 in 4 experiments concerning the influence of

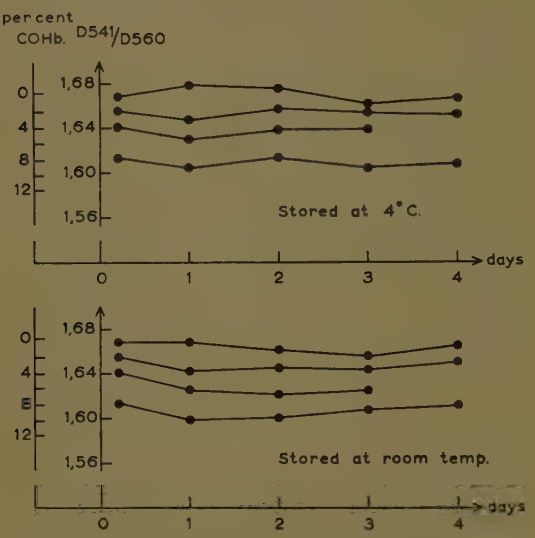

Fig. 2. The results obtained by analysis of blood samples immediately after sampling and after 1, 2, 3 and 4 days of storage in glass capillary tubes at room temperature and 4° C.

storage. In each experiment the subject breathed a mixture of air and CO in a closed circuit system for 30 minutes to attain a steady state with a maximum constant level of HbCO in his blood during sampling. A large number of glass capillary tubes were then filled with ear blood. Analyses were made immediately after sampling and after 1, 2, 3 and 4 days of storage at room temperature and 4° C. The results have not been corrected for MHb. The figure shows that the ratio D 541/D 560 displays only minor changes during these conditions for blood containing 0—8 per cent HbCO. The ratio D 576/D 560 has shown more irregular changes during storage. We, therefore, prefer to use the ratio D 541/D 560 for the determination of HbCO in stored blood. Table III shows the results of analysis of 15 blood samples immediately after sampling and after

Table III. *Carbonylhemoglobin and m hemoglobin in per cent of total hemoglob as determined by spectrophotometric an lysis of 15 blood samples immediately aft sampling and after 2 days of storage at room temperature.*

No storage		Stored 2 days at room temp.		HBCO percentag change during storage
M. HB.	HB CO	M. HB.	HB CO	
0.2	0.0	0.8	1.0	+1.0
0.4	2.0	0.7	3.0	+1.0
0.2	2.5	0.3	2.5	0
0.3	3.0	0.2	2.5	—0.5
0.8	3.5	0.0	3.0	—0.5
0.5	3.5	0.6	4.5	+1.0
0.5	4.0	1.0	4.0	0
0.1	4.0	0.7	4.5	+0.5
0.6	4.0	0.2	2.5	—1.5
0.6	5.5	0.1	6.5	+1.0
0.5	9.0	2.7	8.5	—0.5
0.6	12.5	0.2	12.5	0
0.5	14.5	1.8	15.5	+1.0
0.6	25.0	2.0	25.0	0
0.6	32.0	1.9	31.5	—0.5

2 days of storage at room temperatu (venous blood to which is added a f crystals of sodium citrate, stored in gl tubes under a layer of toluene). MHb w determined in all samples, and the ra D 541/D 560 was corrected for the amou of MHb. Per cent HbCO is read from t calibration curve on the basis of this correct ratio. The table shows no change in perce age HbCO during two days of storage blood containing 0—32 per cent HbCO.

SUMMARY AND CONCLUSIONS

In conclusion, it can be said that the sp trophotometric determination of HbCO blood is sufficiently accurate for an evaluat of the extent of exposure to CO. With use of the technique described, the analy

y be performed after 2—4 days of storage hout interference with the results. The nique is simple, three parallel determina- ns requiring about 5 minutes for analysis.

ACKNOWLEDGEMENTS

The gasometric analyses have been performed H. Steen.

The author is highly indebted to B. Fallentin H. E. Refsum for discussions and valuable ice.

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SIMULTANEOUS CONTRIBUTION TO THE FORMATION OF THROMBIN BY THE INTRINSIC AND EXTRINSIC BLOOD CLOTTING SYSTEMS

By B. A. WAALER

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(Received for publication June 25, 1957)

Proconvertin (factor VII, stable factor) does not contribute to the formation or to the effect of blood thromboplastin (blood prothrombinase) as measured by the thromboplastin generation test, the prothrombin consumption test or the whole blood clotting time (Hicks, 1955; Ackroyd, 1956). The addition of tissue thromboplastin to plasma seems to make blood platelets and the antihemophilic factors superfluous. Proconvertin, however, is essential for coagulation with tissue thromboplastin.

These findings have led to the supposition that there are two different blood clotting systems, termed the *intrinsic* and the *extrinsic* coagulation systems (Bergsagel, 1955). In the intrinsic system blood platelets and the antihemophilic factors, but not proconvertin, are necessary. The extrinsic blood clotting system is intitiated by tissue thromboplastin. For normal function it needs proconvertin but not platelets or the antihemophilic factors. Proaccelerin (factor V, labile factor) and Stuart factor (Hougie, Barrow & Graham, 1957) are necessary for both systems.

When tissue thromboplastin is added to blood or plasma, it is usually presumed that clotting is a result of the extrinsic system alone and that the intrinsic system need not be considered then. Experiments described below demonstrate that this assumption is not always correct. Tissue thromboplastin is shown to initiate *both* clotting systems when added to normal plasma. The two clotting systems may generate thrombin simultaneously, the clotting time thus being a resultant of the activities of both systems.

REAGENTS

Normal human platelet-poor citrated plasma. A clean venepuncture was made with a 19 gauge needle coated with Arquad 2 C (Armour). The first five ml of blood were discarded, and nine volumes of blood were then collected directly into a silicone-coated, ice-cooled centrifuge tube, containing one volume 3.13 g per cent sodium citrate dihydrate. The blood was immediately centrifuged for 30 minutes at 2 500 r. p. m. at 4° C. The plasma was transferred to lusteroid tubes with a siliconized pipette and centrifuged for one hour at 20 000 r.p.m. at low temperature. The platelet-poor plasma was collected, discarding the lipoid-rich top-layer. The plasma was stored at — 20° C in stoppered lusteroid tubes.

Platelet-poor citrated hemophilia A plasma was obtained from a patient with severe hemophilia A, the coagulation defect being corrected by the addition of dilute, barium sulfate-adsorbed oxalated bovine plasma, but not by the addition of stored serum.

Platelet-poor citrated hemophilia B plasma was ained from a patient with severe hemophilia B C deficiency, Christmas disease), the coagula- defect being corrected by the addition of dilute ed serum, but not by the addition of adsorbed ne plasma.

Platelet-poor citrated proconvertin-deficient plas- was obtained from a patient with a congenital ciency of proconvertin. The plasma had normal cent of prothrombin and proaccelerin and less n 1 per cent of proconvertin as measured by our hods. The other properties of this plasma were lar to those of the patients described by Owren 53).

The hemophilia A plasma, the hemophilia B ma and the proconvertin-deficient plasma were drawn, treated and stored as described for the mal plasma. New samples were thawed for each eriment, used within three hours after thawing, during this time kept at 4° C.

Adsorbed bovine plasma. Nine volumes of blood e mixed with one volume 2.5 g per cent potas- n oxalate monohydrate and cooled to 4° C. The d was centrifuged for one hour at 2 500 r.p.m. plasma was then adsorbed with 100 mg barium ate (Baker) per ml for 15 minutes and centri- ed for one hour at 2 500 r.p.m. Finally, the ma was dialyzed against a solution of 0.9 g cent sodium chloride overnight. The plasma was ed at —20° C in convenient aliquots.

Tissue thromboplastin. A saline-buffer suspen- of human brain was prepared and stored as ribed by Hjort (1957). All dilutions of the k suspension were made in saline-buffer. The entration which was optimal with normal elet-poor plasma in a Quicksystem is referred s 100 per cent.

Crude cephalin suspension was prepared from an brain as described by Hjort, Rapaport & ren (1955) and kept in the liquid frozen state —20° C. The stock suspension was diluted in e-buffer. A 1 : 200 dilution of the stock suspen- was optimal with the normal platelet-poor ma and is referred to as 100 per cent con- ration.

Proconvertin reagent was prepared from the serum of a patient with hemophilia B by the technique described by Hjort (1957). The reagent was dissolved in saline-buffer to one half the volume of the serum from which it was prepared. The reagent contained 130 per cent proconvertin and gave only a slight shortening of the recalcification time of hemophilia B plasma.

Anthihemophilic B factor reagent was prepared from the serum of a patient with congenital proconvertin deficiency. The same technic was used as for the proconvertin reagent. The reagent contained less than ½ per cent prothrombin. When tested in our proconvertin system it appeared to contain about 1.5 per cent proconvertin activity, Measured in a special system for antihemophilic B factor (Stapp 1957) the reagent contained about 32 per cent of this factor.

Veronal buffer of pH 7.35 and ionic strength 0.154 was prepared as described by Owren (1947, p. 125).

Saline-buffer mixture consisted of one volume veronal buffer to nine volumes of 0.9 g per cent sodium chloride solution.

Dilution fluids for the P and P method. The test plasma was first diluted in *dilution fluid I* to 10 times the final concentration desired. This dilution fluid consists of 100 ml of 3.13 g per cent sodium citrate dihydrate solution to 500 ml of 0.9 g per cent sodium chloride solution, and it has nearly the same citrate concentration and ionic strength as citrated plasma from blood with normal hematocrit. The final 1 : 10 dilution was made in *dilution fluid II,* which consists of 200 ml of veronal buffer, 200 ml of 25.66 mM sodium citrate dihydrate solution and 600 ml of 0.9 g per cent sodium chloride solution, and has a pH of 7.35 and an ionic strength of 0.154.

METHODS

The cephalin time. The following procedure was used: 0.2 ml of the testplasma, 0.2 ml of a cephalin suspension and 0.2 ml of either saline-buffer or a reagent diluted in saline-buffer were mixed, in-

cubated for 6 minutes at 37° C, and then recalcified with 0.2 ml of an optimal solution of $CaCl_2$ (usually 25 mM). After the recalcification the tube was tilted gently four times and then replaced in the water bath. At intervals the tube was inspected with the help of cross illumination against a black background while being rotated (not tilted) gently. The cephalin time was measured as the interval between the addition of $CaCl_2$ and the formation of the first threads of fibrin. The approximate cephalin time was first found in a pilot test. The accurate cephalin time was then estimated by duplicate tests, in which the tubes were left undisturbed until 6—7 seconds before the end point found in the pilot test.

It is essential for the accuracy of this method to use clean, clear, faultless tubes and to standardize the performance of the test as described. It is also important to use a constant incubation time of 6 minutes. When carried out as described, the method gave much more reproducible results than the usual recording of solid clot formation as seen when tilting the tubes.

The thromboplastin time was estimated by the same technic, using a suspension of tissue thromboplastin instead of cephalin.

The recalcification time was also estimated in the same way, using 0.2 ml saline-buffer instead of cephalin.

Prothrombin was measured by the method of Hjort *et al.* (1955).

Proconvertin was measured by the method described by Aas (1952), using plasma from a patient with proconvertin deficiency as substrate.

All tests were run at least in duplicate. Selected, faultless glass tubes with an internal diameter of 8 mm were used.

General Electric Dri Film 9987 was used for the silicone coating of the collection tubes. Each tube was siliconized twice.

RESULTS

The tissue thromboplastin time of a proconvertin deficient plasma is grossly prolonged. If such a plasma was tested with different concentrations of tissue thromboplastin, it reacted quite differently than a normal plasma, as shown in Fig. 1.

If the two plasmas were tested with cephalin suspensions of varying strengths, however, the curves were similar (Fig. 2).

The curves for various dilutions of tissue thromboplastin and for various dilutions of cephalin with proconvertin deficient plasma had exactly the same shape (Fig. 3).

Optimal concentrations of tissue thromboplastin and of cephalin gave the same clotting times with this plasma, whereas there were marked differences between the corresponding times in a normal plasma (Fig. 1 and Fig. 2). If proconvertin reagent was added to the proconvertin deficient plasma, its reaction with tissue thromboplastin became the same as that of the normal plasma (Fig. 4), whereas its reaction to cephalin was nearly unchanged.

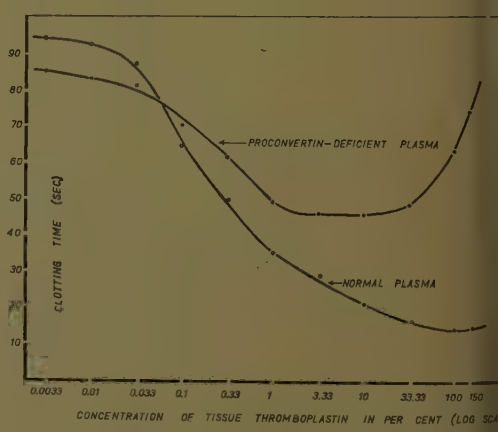
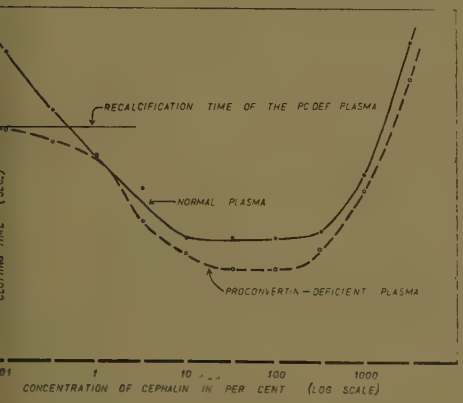
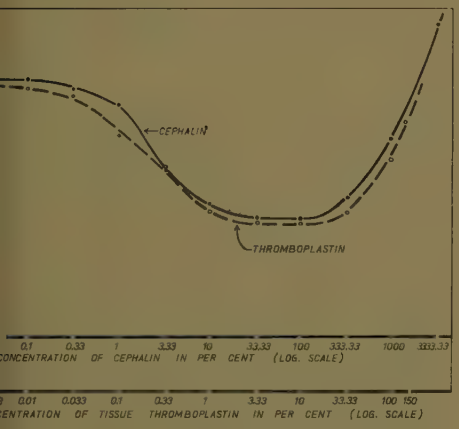


Fig. 1. Clotting times of normal platelet-poo plasma and of proconvertin-deficient platelet-poo plasma with varying concentrations of tissue thron boplastin. (The thromboplastin concentration whic was optimal with normal plasma was chosen the 100 per cent value).



2. Clotting times of normal platelet-poor
a and of proconvertin-deficient platelet-poor
a with varying concentrations of cephalin.
cification time of the normal plasma: 118
ds. Recalcification time of the proconvertin-
deficient plasma: 87 seconds.


CEPHALIN
THROMBOPLASTIN
0.1 0.33 1 3.33 10 33.33 100 333.33 1000
CONCENTRATION OF CEPHALIN IN PER CENT (LOG. SCALE)
0.01 0.033 0.1 0.33 1 3.33 10 33.33 100 150
ENTRATION OF TISSUE THROMBOPLASTIN IN PER CENT (LOG. SCALE)

. Clotting times of platelet-poor proconvertin-
ent plasma with varying concentrations of
in and with varying concentrations of
thromboplastin. (The concentrations of ce-
and of tissue thromboplastin which were
mal with normal plasma were chosen as
100 per cent values).

ese findings suggest that the effect of
thromboplastin on proconvertin defi-

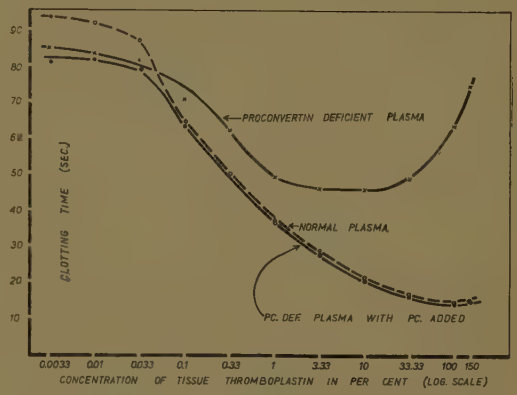


Fig. 4. Clotting times with varying concentrations of tissue thromboplastin in platelet-poor proconvertin-deficient plasma, in platelet-poor normal plasma, and in proconvertin-deficient plasma after the addition of proconvertin. Diluted proconvertin reagent (1 : 2), containing about 60 per cent activity, was added. Pc. = proconvertin.

cient plasma is identical to that of cephalin ("partial thromboplastin") : initiation of the intrinsic clotting system. It must be assumed therefore that tissue thromboplastin — by its cephalin like effect — also in normal plasma initiates the intrinsic clotting system.

Fig. 5 demonstrates that the two clotting systems actually *can* run simultaneously in the same plasma. It illustrates the reaction of a normal plasma with various concentrations of tissue thromboplastin both with and without the addition of a cephalin suspension of constant and optimal concentration. At high concentrations of tissue thromboplastin the addition of cephalin was without influence. When the concentration of thromboplastin was lowered, however, an influence from the added cephalin showed up. With concentrations of tissue thromboplastin between 10 per cent and 0.033 per cent, both clotting systems contributed to coagulation since the times

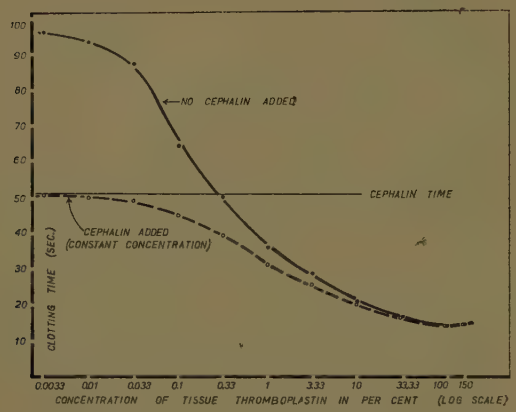

NO CEPHALIN ADDED
CEPHALIN TIME
CEPHALIN ADDED (CONSTANT CONCENTRATION)
CLOTTING TIME (SEC.)
CONCENTRATION OF TISSUE THROMBOPLASTIN IN PER CENT (LOG SCALE)

Fig. 5. Clotting times of normal platelet-poor plasma with varying concentrations of tissue thromboplastin, with and without the addition of a cephalin suspension of constant, optimal concentration. Clotting system: 0.2 ml plasma, 0.2 ml tissue thromboplastin (varying concentrations), 0.2 ml cephalinsuspension (constant concentration — 100 per cent) *or* 0.2 ml saline-buffer, and 0.2 ml 25 mM $CaCl_2$ solution.

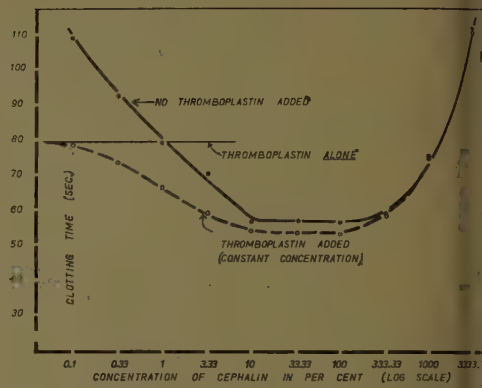
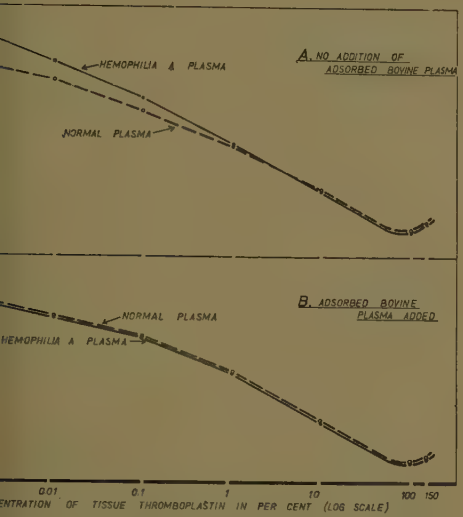

NO THROMBOPLASTIN ADDED
THROMBOPLASTIN ALONE
THROMBOPLASTIN ADDED (CONSTANT CONCENTRATION)
CLOTTING TIME (SEC.)
CONCENTRATION OF CEPHALIN IN PER CENT (LOG SCALE)

Fig. 6. Clotting times of normal platelet-p plasma with varying concentrations of cephalin, w and without the addition of diluted tissue thro boplastin of constant concentration (0.066 per ce Clotting system: 0.2 ml plasma, 0.2 ml cephalin-s pension (varying concentration), 0.2 ml diluted t sue thromboplastin (constant concentration — 0.0 per cent) *or* 0.2 ml saline-buffer, and 0.2 ml 25 m $CaCl_2$ solution.

were shorter than those found with tissue thromboplastin or cephalin alone. At concentrations of tissue thromboplastin lower than 0.033 per cent, the clotting time of the plasma equaled the cephalin time. This probably indicates that coagulation then was due only to the intrinsic clotting system.

Fig. 6 shows another situation. Cephalin suspension of *varying* concentration and a dilute tissue thromboplastin of *constant* concentration were added to a normal plasma. The figure indicates a simultaneous contribution to the clotting by both cephalin and tissue thromboplastin. At the optimal cephalin concentrations the plasma clotted mainly by the action of cephalin on the intrinsic clotting system. Addition of the weak solution of tissue thromboplastin gave only a 3-second shortening of the clotting time. With i creasing dilution of the cephalin suspensio the influence of tissue thromboplastin a the extrinsic blood clotting system gradua increased. At concentrations below 0.33 p cent the cephalin suspension was too we to influence the clotting time.

In hemophilia A plasma the extrinsic cl ting system is normal, whereas the intrin system is highly defective. Fig. 7 A. sho the clotting times of such plasma with tiss thromboplastin of various concentrations compared with normal plasma. It confir the finding of Dam & Venndt (1940), Bi & Macfarlane (1951) and others that hem philic plasma reacts similarly to norm plasma with concentrated tissue throm plastin, but shows a relative "resistance"



7. Clotting times of platelet-poor normal and et-poor hemophilia A plasma with varying ntrations of tissue thromboplastin. A. Without addition to the test plasmas. B. With addition 1 : 5 dilution (about 40 per cent antihemophilic r A activity) of adsorbed bovine plasma to both lasmas. Clotting system: 0.2 ml plasma, 0.2 ml ion fluid II or 0.2 ml of a 1 : 5 dilution of the bed bovine plasma in dilution fluid II, 0.2 ml thromboplastin (varying concentration) and 0.2 ml 25 mM $CaCl_2$ solution.

e thromboplastin. If the hemophilic de- was corrected by the addition of ad- ed bovine plasma, diluted 1 : 5 in saline- er, the "resistance" disappeared, and the ophilic plasma reacted with tissue throm- astin as did the normal plasma (Fig. . An amount of proaccelerin which ld accelerate both the intrinsic and the insic blood clotting system was also d with the diluted bovine plasma.

rom these various experiments it may be luded that tissue thromboplastin acts like alin on the intrinsic clotting system. clotting systems therefore are initiated ltaneously by tissue thromboplastin. Consequently the intrinsic system may influence various tests in which tissue thromboplastin is used as a reagent. Although the extrinsic system usually dominates such tests, the intrinsic system becomes important when the extrinsic system is defective. As an example, the P and P method of Owren (Owren &Aas, 1951) was analyzed. This method has been thought to measure the combined effect of prothrombin and proconvertin. Seitz-filtered bovine plasma, human brain thromboplastin and calcium chloride are the reagents. Fig. 8 No. 2 gives the clotting times for dilutions of normal plasma in the usual P and P system. A 1 : 10 dilution of the plasma in dilution fluid II serves as the 100 per cent value. For values between 100 and 20 per cent the curve gives a straight line on double logarithmic paper. Below 20 per cent it gives a somewhat steeper straight line. The corresponding dilution curve for hemophilia B plasma is parallel with that of the normal plasma but the times are prolonged — about 4 seconds at the 100 per cent level (Fig. 8 No. 1).

The hemophilia B plasma contained about the same concentration of prothrombin and proconvertin as the normal plasma (Table I).

If the hemophilia B plasma was normalized by the addition of antihemophilic B factor reagent, it gave exactly the same dilution curve as the normal plasma (Fig. 8 No. 3).

The only source of prothrombin, proconvertin and antihemophilic B factor in the P and P system is the test plasma. When a normal plasma is tested all three factors are diluted to the same degree. By keeping the concentration of the antihemophilic B factor constant (96 per cent activity) in all dilutions, the effect of prothrombin and procon-

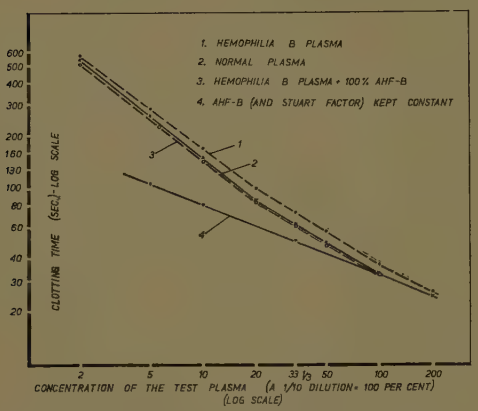


Fig. 8. The influence of adsorbable clotting factors other than prothrombin and proconvertin on the clotting times in the P and P method. Progressive dilutions of the following test plasmas were tested with the P and P method: 1. Platelet-poor hemophilia B plasma. 2. Platelet-poor normal plasma. 3. Platelet-poor hemophilia B plasma after normalization of the 1 : 10 plasma dilution (the 100 per cent value) with antihemophilic B factor reagent. Further dilutions with dilution fluid II. 4. Platelet-poor hemophilia B plasma with a constant amount of antihemophilic B factor reagent added to each dilution of the plasma. The amount of antihemophilic B factor was thus kept constant (at about 100 per cent) in all plasma dilutions.
AHF-B = antihemophilic B factor.

vertin alone could be studied. This was achieved by doing all the final 1 : 10 dilutions of the hemophilia B plasma in a mixture of 0.6 ml antihemophilic factor B reagent and 1.2 ml dilution fluid II. The dilution curve then became less steep than that of the normal plama and gave a straight line from 100 per cent to 5 per cent (Fig. 8 No. 4).

DISCUSSION

The findings reported support the theory of two different blood clotting systems. The intrinsic blood clotting system, as measured by the cephalin time and the recalcification

Table I. *The concentration of prothromb and proconvertin as measured by speci methods, and the P and P values of platel poor normal plasma and of platelet-po hemophilia B plasma.*

	Pro-thrombin (per cent)	Procon-vertin (per cent)	PP-valu (per ce
Normal plasma	101	100	100
Hemophilia B plasma	100	94	82

time, was found to be normal in proconvert deficient plasma. The clotting times of th plasma with various dilutions of tissue thro boplastin and with various dilutions of ceph lin gave identical curves. The optimal dil tion of tissue thromboplastin clotted the pr convertin deficient plasma in the same ti as did the optimal dilution of cephalin. T optimal concentration of thromboplast however, was only $^1/_{10}$—$^1/_{30}$ of that optim for normal plasma, where the reaction wi various dilutions of tissue thromboplast gave quite another curve. The addition proconvertin to the deficient plasma chang its thromboplastin curve to normal.

These experiments indicate that tiss thromboplastin from human brain acts in o way in the presence of proconvertin and quite another way in the absence of th factor. When proconvertin is absent, the fect of tissue thromboplastin equals that cephalin, and it is reasonable to believe th this effect is mediated through the intrin blood clotting system. Traces of proconver may be present in the deficient plasma, t the possibility that the tissue thromboplas here should act through the extrinsic syst seems unlikely from the findings present above.

hen added to a normal plasma tissue
boplastin, contrary to cephalin, initiated
clotting systems. When concentrated
thromboplastin is used, however, the
rapid extrinsic system will dominate
nd result. That both systems *can* run
taneously is shown in the experiments
hich both cephalin and tissue thrombo-
n were added to a normal plasma, one
e reagents being kept constant, while
ther was progressively diluted. When
thromboplastin was used with cepha-
ne clotting times were shorter than with
of the reagents alone, indicating that
clotting systems contributed to the coa-
ion of the plasma. In these experiments
nfluence of the relatively slow-acting in-
c system is only seen with clotting times
r than 20—30 seconds.

e socalled "resistance" of hemophilic
a to dilute tissue thromboplastin is only
lection of the defective intrinsic clotting
m. One must presume that both clotting
ms generate thrombin simultaneously,
ting in the formation of fibrin when suf-
t amounts of thrombin have accumul-
When concentrated (optimal) tissue
boplastin is used, however, the extrinsic
m, both in normal and in hemophilic
a, will generate thrombin so rapidly
his system alone determines the clotting
When dilute tissue thromboplastin is
however, giving prolonged clotting
, the intrinsic system will add to the
bin formation in a normal plasma. In
nophilic plasma this contribution from
trinsic system is lacking, and the clot-
time consequently becomes longer than
normal plasma. After correction of the
philic defect, there is no difference in
the response of a normal plasma and a hemophilic plasma to dilute tissue thromboplastin.

It has some practical importance that clotting tests using tissue thromboplastin as a reagent may be influenced by factors also from the intrinsic system. The P and P test, originally devised to measure only prothrombin and proconvertin, is in fact to some degree influenced by the content of antihemophilic B factor in the test plasma. When antihemophilic B factor varies in parallel with prothrombin and proconvertin, the dilution curve is steeper than when the first factor is kept at a constant level. This indicates an increasing sensitivity to antihemophilic B factor with increasing clotting times.

The P and P method is probably sensitive also to another adsorbable factor, namely the Stuart factor (Hougie *et al.*, 1957). Our antihemophilic B factor reagent probably contained Stuart factor, which was then also kept at constant concentration in curve No. 4, Fig. 8. The shortening of the clotting times in this curve might thus partly be due to the effect of the Stuart factor. Lacking a method for its estimation we have been unable to evaluate the relative importance of this factor in the P and P test.

It has been shown that the antihemophilic B factor (Sise, Kimball & Adamis, 1955; Naeye, 1956; Verstraete & Vandenbroucke, 1956; Stapp, 1957) and Stuart factor (Hougie *et al.*, 1957) are lowered during anticoagulant treatment with dicumarol and similar drugs. The lowering of these factors is probably at least partly responsible for the bleeding tendency during treatment. The very low frequency of bleeding episodes reported in groups of anticoagulant treated patients controlled with the P and P method may

thus be explained by the fact that this method also reflects lowering of the antihemophilic B factor and the Stuart factor. This sensitivity of the P and P method increases its usefulness as a test for control of anticoagulant therapy.

SUMMARY

1. In proconvertin deficient plasma, tissue thromboplastin acts as cephalin (partial thromboplastin) and initiates the normal intrinsic system of this plasma.

2. In normal plasma, tissue thromboplastin initiates both the extrinsic and the intrinsic clotting systems. With concentrated tissue thromboplastin the more rapidly proceeding extrinsic system dominates thrombin formation and is decisive for the clotting time. With dilute tissue thromboplastin or when the extrinsic system is partly defective, the intrinsic system may, however, add to the thrombin formation and thereby influence the clotting time.

3. The "resistance" of hemophilic plasma to dilute tissue thromboplastin is only a reflection of the defective intrinsic clotting system which is unable to contribute to thrombin formation.

4. The P and P method of Owren is influenced by the content of antihemophilic B factor and Stuart factor in the testplasma.

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BLOOD PRESSURE AND URINARY EXCRETION OF NOR-ADRENALINE (NA)

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ithin the field of research on hyperten- the kidneys have for many years been e focus of attention both experimentally clinically. However, during the latest le a number of significant extrarenal rs of neurogen as well as humoral na- have been disclosed. As far as the latter are concerned, the hormones of the al cortex as well as the adrenal medulla been of particular interest.

1949 Goldenberg, Apgar, Deterling & s showed that nor-adrenaline (NA) uces an elevation in the systolic as well e diastolic blood pressure by increasing eripheral resistance. A few years earlier l been established by von Euler (1946) NA along with adrenaline is the humoral mitter of the sympathetic nervous m and can be demonstrated in the or- m corresponding to this system, thus g others in the adrenal medulla (Holtz, ner & Kroneberg, 1947).

oof of NA as active pressor substance n the normal physiological pattern ne- rily involves this factor in the research ypertension. Nature has furnished a physiological proof of the role of NA at pheochromocytomas may give symp- of malignant hypertension while at the time large quantities of NA may be found in blood and urine. In some instances of pheochromocytoma the hypertension may be paroxysmal indeed, but most frequently the hypertension is persistent (Green, 1946) and may in many cases disappear after operative removal of the pheochromocytoma. From the clinic it is further known that by permanent infusion of NA, any level of blood pressure may be maintained, also a hypertensive one. Goldenberg, Pines, Baldwin, Green & Roh (1948) have shown that at any rate in some hypertensives there is an increased sensitivity to NA.

The urinary excretion of NA gives only an imperfect impression of the role of NA in the actual blood pressure. The formation of NA, destruction of NA, sensitivity to NA, and the mode of reaction in the interrelationship with the other factors regulating the blood pressure will be unknown quantities even if the urinary excretion of NA has been established. The estimation of the NA excretion in urine is, however, one of the few methods we have at disposal within this field for the time being and such determinations have at any rate proved of great importance for the diagnosis of pheochromocytomas.

Only from 1 to 4 per cent of infused NA is excreted in urine (von Euler, 1956). The

normal excretion per 24 hours is about 50 μg. The excretion may be increased considerably by hard muscular work (von Euler & Hellner, 1952). The excretion in cases of pheochromocytoma as a rule assumes quite another order of magnitude (1—2 mg per 24 hours).

But few investigations on NA-excretion in essential hypertension are published. Goldenberg (1954), by biological analysis of nor-adrenaline plus adrenaline in 35 hypertensive patients, found from 7 to 100 μg/24 h calculated as NA. In 13 normal cases the findings were from 14 to 41 μg/24 h. Von Euler & Hellner (1951) found an average excretion of 30 μg/24 h in 20 healthy medical students ranging from 9.6 to 63 μg/24 h NA. In a comprehensive material of 500 cases of essential hypertension von Euler, Hellner & Purkhold (1954), likewise by biological analysis, found: 66 per cent "normal" excretion (less than 57.6 μg/24 h) 17.4 per cent with excretion ranging from 57.6 to 86.4 μg/24 h, and 16.4 per cent with excretion above 86.4 μg/24 h. Only the latter group was considered absolutely above normal excretion. Burn (1953) in a small material found no certain difference between hypertensives and normal subjects.

Below is given an account of a comparison between the level of blood pressure and the NA excretion in 100 patients with normal as well as increased blood pressure.

MATERIAL

From the files of the laboratory a random sample of 100 NA determinations in urine[1] was selected. For these patients information on bl pressure and diagnosis and — in cases where was possible — on blood urea, urine analysis funduscopic examinations, was collected.

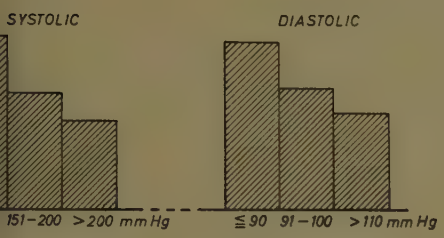
The blood pressure which was put in relat to the urinary excretion of NA was the stabiliz or final pressure during the stay at hospital. I few instances where the blood pressure has b variable the blood pressure at the time of col tion of urine has been chosen in so far as this been possible. In 25 cases the diagnosis was hyp tensio arterialis only, in 24 cases hypertension another diagnosis such as encephalopathia, a plexia, mb. cordis, diabetes, neurosis. In four ca the diagnosis was hypertension plus renal disc in two cases renal disease only. In the remain 39 cases (all normotensive) the diagnosis was fa evenly distributed among cases such as mb. cor lipothymia, vertigo, paroxystic tachycardia, arte sclerosis cerebri, epilepsy, cephalalgia, neuro vagus syndrome, and in some of these instances the diagnosis observation for pheochromocyt (not found). Two cases of pheochromocytoma v fied by operation were excluded from the ma rial. For further characterization of the mate it may be added that in 59 cases information ab examination of the urine for albumin was giv of these 9 were + a, 2 ± a, and 48 — a.

Blood urea was known in 51 cases, increa in only 3 of these (above 50 mg per cent). In cases ophthalmoscopy was performed (see belo

METHODS

The method of Lund (1949, 1950) designed determination of A and NA in blood was u A modification of the method was necessary ow to the fact that the reproducible recovery at 7 80 per cent in serum analysis was not found w analysing urine. The specific fluorescence of and NA varies from 20 to 90 per cent in vari samples of urine either on account of quenchi effect (the ability of certain components of u to inhibit the fluorescence) or on account of va ing adsorption on aluminum oxide caused by v able contents of salts in the urine. For this rea it was necessary to make recovery tests with individual urine and correct the spontaneous va measured accordingly.

[1] The urine specimens were sent from different hospitals in Denmark, without case reports or diagnosis, for quantitative NA analysis.



1. Average 24 hours urinary excretion of NA n three levels of blood pressure (systolic and diastolic).

Lund's original method A and (A + NA) letermined. NA is calculated as a difference consequently liable to errors of both deter- ions. As it has appeared that the ratio between d NA does not vary much and A makes up atively small part (about 17 per cent) of the adrenaline when dealing with excretion of al and subnormal quantities, it has been pre- d for the purpose of the present work to make tive determinations of A and NA with a view ducing the error in these figures which before- are so small. The result, in accordance with bove-mentioned, represents predominantly NA.

RESULTS

The material is divided into three groups according to the level (mm Hg) of the systolic and diastolic blood pressure. Systolic: $\leqq 150$ (A), 151—200 (B) and > 200 (C). Diastolic: $\leqq 90$ (a), 91—110 (b) and > 110 (c). Diagram 1 shows the average excretion of NA within the three groups.

The statistical treatment of the material has been made on the basis of Fischer's formulae (1948) and is presented in Tables I and II.

P-values below 1 per cent mean that there is > 99 per cent possibility of the difference not being accidental. With a P-value of 5 per cent as significance limit, the differences between groups A and B and between A and C as well as between a and b and a and c are significant. The conclusion is that patients with increased blood pressure have a significantly lower NA excretion than patients with normal blood pressure. Besides, a marked tendency to lower NA excretion

Table I. *24 hours urinary excretion of NA within three levels of systolic blood pressure.*

	Number of individuals (n)	Excretion of NA µg/24 h Mean (M)	Standard error of the mean (e_M)	Difference of means	Standard error of difference (e_{diff})	t	P
p A							
) mm Hg	38	64	5.25				
p B							
-200 mm Hg	38	43	3.73				
p C							
) mm Hg	18	33	4.59				
p A—B				21	6.44	3.26	< 1 per cent
p B—C				10	5.92	1.69	<10 »
p A—C				31	6.97	4.45	< 1 »

Table II. *24 hours urinary excretion of NA within three levels of diastolic blood pressure.*

	Number of individuals (n)	Excretion of NA µg/24 h Mean (M)	Standard error of the mean (e_M)	Difference of means	Standard error of difference (e_{diff})	t	P
Group a ≦90 mm Hg	43	61	4.48				
Group b 91—110 mm Hg	23	45	6.11				
Group c >110 mm Hg	28	36	4.22				
Group a—b				16	7.58	2.11	< 5 per ce
Group b—c				9	7.42	1.21	<30 »
Group a—c				25	6.15	4.06	< 1 »

with rising blood pressure is seen even if the difference between the groups of elevated blood pressure B—C and b—c is not significant at the 95 per cent band.

Ophthalmoscopy was performed in 53 cases and the material grouped according to retinal changes in 1—4 grades. The average excretion of NA in the different groups was: Normal (19 cases) 59 µg/24 h, grade 1 and 1—2 (15 cases) 60 µg/24 h, grade 2 and 2—3 (14 cases) 42 µg/24 h, grade 3 and 3—4 (5 cases) 43 µg/24 h.

The comparison between the hypertensive retinal changes and the NA excretion further illustrates the tendency: that the NA excretion varies inversely to the degree of hypertension.

DISCUSSION

The statistically significant difference proved between the various groups of blood pressure is the more noteworthy in that it has been found by means of a method that as a matter of routine is used as a screen test only for determination of the large quantities of NA and A excreted in cases of pheochromocytoma. From this it must justifiable to conclude that the negative co relation between blood pressure and NA e cretion must be very pronounced. The ci cumstance that this difference has not be proved at an earlier time may be due to predominant search for increased NA e cretion in hypertension which, indeed, wou be naturally expected from the experienc with pheochromocytoma. Only if one realiz that the NA excretion is reduced in hype tension and divides the material as show does the correlation become apparent.

The result of this present study seems be contrary to that of von Euler & Helln These authors in a material comprising 5 hypertensive patients found a group (16 per cent) with a NA excretion above norm To this must be said, however, that the was no classification of the cases of hyp tension in the von Euler & Hellner materi thus in particular no comparison between t level of blood pressure and the NA exc tion. Further no information is given ab the diastolic blood pressure; we only kn that in all instances the systolic blood pr

was above 150 mm, and that the diagno- n all cases was hypertension. Thus it is excluded that the cases with high NA etion had normal diastolic or mean blood sure. A more recent Swedish work on excretion based on the von Euler tech- e (Sundin, 1956) has been published. The lts of this work are in line with those ne present investigation. Sundin found the NA excretion in recumbent posi- was somewhat lower in hypertensive nts than in normal subjects. When ed with the head up at an angle of 75° n the horizontal plane the NA excretion considerably in normal subjects. In rtensive patients this increase of the excretion was significantly lower (hyper- ves 13.7, normal subjects 34.1 millimi- rammes per minute on an average).

this connection it can be said against present material that the orthostatic in- nces on the NA excretion have not been stered. However, it has been impossible he basis of the case records to establish her all the patients have been confined ed during collection of the urine. How- , on the basis of the distribution of diag- s within the three groups, one may as- e that any essential difference between ment of the groups as regards confine- t to bed is not very likely.

s in spite of all it cannot be left out of ideration that the matter of confinement ed and muscular work may have played rt in this present material, we have made ntrol investigation comprising 30 patients ined to bed. All these patients had been italized in the same ward and subjected e same routine. In this material we have n blood pressure measured on the sixth

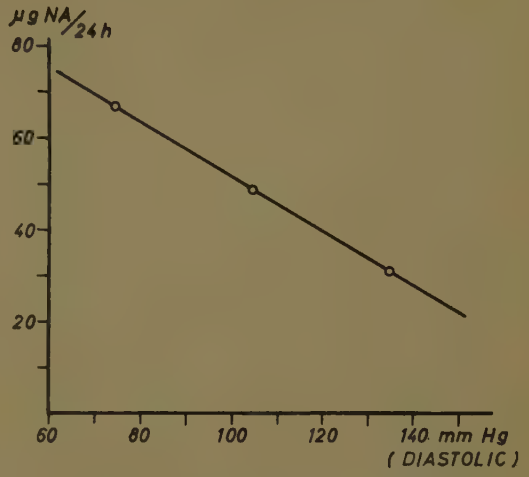


Fig. 2. Average 24 hours urinary excretion of NA in 30 patients (all confined to bed) grouped after diastolic blood pressure in three classes, with intervals of 30 mm Hg.

or seventh day after admission as the collection of the 24 hours urine for NA analysis was also made at that time. All patients were examined at the same ophthalmologic clinic. The diagnosis in almost all cases was hypertensio arterialis. Apart from one case the urine was — alb. The blood urea has been examined in 26 patients and was in all instances below 50 mg per cent. Owing to the smallness of the material we have not made any statistical treatment but have shown the relationship between blood pressure and NA excretion in a *diagram (2)*.

The diagram clearly illustrates the same tendency as in the larger material. The average NA excretion in these patients grouped after retinal changes was: Normal (12 cases) 54 µg/24 h, grade 1 and 2 (13 cases) 52 µg/24 h, grade 3 and 4 (5 cases) 32 µg/24 h.

The theoretical explanation of these results cannot be given at the present time but some possibilities shall be briefly mentioned.

It may be thought that hypertensives excrete substances with quenching effect and that the excretion is dependent on the gravity of the disease. As, however, as mentioned, recovery tests have been made with each individual sample of urine, *i. e.* determination of the fluorescence of a known quantity of NA in the said milieu and the determinations have been used for the calculation of the results reported, this possibility may be left out. An explanation close at hand would be renal failure, *i. e.* reduced NA clearance in the severe cases of hypertension. Unfortunately practically no examinations of the renal function are available for these patients. The circumstance that only 18 per cent of the patients had albuminuria, that the blood urea was increased (above 50 mg per cent) in hardly 6 per cent, does not indicate that renal disease was dominant in the material. A third possibility is that the decreasing NA excretion with increasing hypertension is a compensatory phenomenon so that the production or liberation of NA is reduced parallel to the increase of the blood pressure. An abnormally fast destruction of A and NA is also within the bounds of possibility. Sundin, whose results are in line with ours, puts forth the hypothesis that the orthostatic reflexes *via* the baroceptive zones of sinus caroticus and aorta are inhibited in hypertensives owing to the fact that the blood pressure is elevated beforehand. The inhibition becomes manifest as a reduction of the endogenous NA production and may be demonstrated as a reduced NA excretion.

To be able to evaluate the perspectives opened by these investigations it will be necessary to supplement the urine determinations with blood analyses. As the concentration of NA in the blood amounts fractions of a microgramme per 100 ml th will require a very sensitive and speci method and technical equipment.

SUMMARY

Blood pressure was compared with urina excretion of NA in 130 patients (normote sive and hypertensive), cases of pheochr mocytoma not included. The excretion NA was found to be reduced in hyperte sion. The difference in NA excretion t tween hypertensive and normal subjects statistically significant and there seems to a negative correlation between the severi of the disease (as measured by the height blood pressure and the grade of retiniti and the excretion of NA.

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IMPROVED SEPARATION OF SERUM PROTEINS IN PAPER ELECTROPHORESIS — A NEW ELECTROPHORESIS BUFFER

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Electrophoretic separation of serum proteins usually yields five main fractions: albumin, α_1-, α_2-, β- and γ-globulin. However, serum contains a much larger number of individual proteins. Incomplete separation is due to several factors, such as insufficient differences in the electrophoretic mobility of the components, diffusion and convection phenomena, the quantitative dominance of some fractions and the difficulty of finding an ideal supporting medium in zone electrophoresis. Attempts have been made to obtain a better separation by varying the experimental conditions. Several ways have been tried, for example varying the potential gradient (Kawerau, 1954; Wiedermann, 1953; Werner & Westphal, 1955), the buffer system (Saifer & Corey, 1955; Hauser & Lynn, 1950; Consden & Powell, 1955; Laurell, Laurell & Skoog, 1956), and the supporting medium in zone electrophoresis (Wunderly, 1956; Ressler & Jacobssen, 1955; Ressler & Zak, 1956; Smithies, 1955; Kohn, 1957). A buffer will be described below in which serum proteins regularly separate into nine fractions.

METHODS

The electrophoresis apparatus used here was constructed according to the principles of the LKB apparatus (Valmet & Svensson, 1954). Three types of paper were tried as supporting medium: filt papers "Whatman I", "Schleicher & Schüll 2043 acetylated", and a cellulose acetate paper fro OXO Ltd., London (Kohn, 1957). The strips we dipped into the buffer solution and blotted befo being placed in the apparatus. The proteins we stained with Amido-Black 10 B (Bayer) (Gras man, Hannig & Knedel, 1951). The strips we evaluated with Kelab's photometer according Köiw (1953) or the direct intergrating phot meter Analytrol from the Spinco Division.

With the filter papers "Whatman I" a "Schleicher & Schüll 2043 B acetylated" the fo lowing buffer which has pH 8.9 was used:

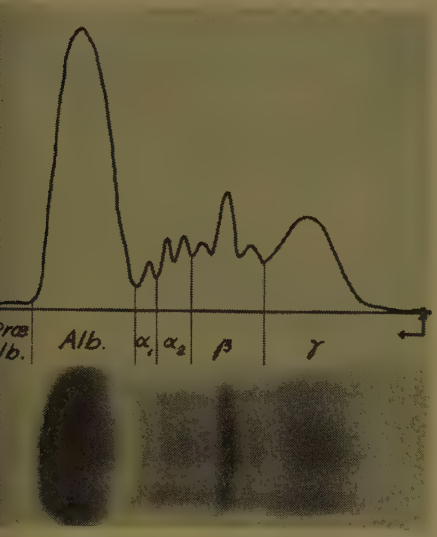
Trishydroxymethylaminomethane (TRIS) 60 g/1 (0.5 mol). Ethylenediaminetetracetic ac (EDTA) 6.0 g/1 (0.021 mol). Boric acid 4 (0.075 mol).

The potential gradient was 6.5 V/cm and t experiments were run for 16 hours.

With the cellulose acetate paper the same buff was 1.3 times more concentrated and the potenti gradient was 4.5 V/cm for 16 hours.

RESULTS

Notable results have been obtained wi the TRIS buffer in the chromatograph separation of serum proteins on ionic e change columns (Sober, Gutter, Wyckoff Peterson, 1956). The advantage of this bu fer is that it has low conductivity at relative high concentrations and in addition a stro buffer effect within the pH range 7— TRIS alone (pH 10.4) or TRIS in co



1. Normal serum separated on a cellulose te strip and the corresponding protein dia- gram evaluated with Köiw's photometer. Note the prealbumin fraction.

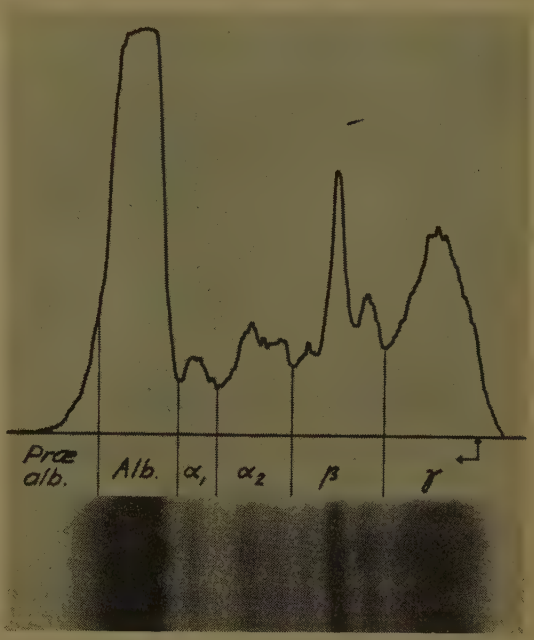


Fig. 2. Normal serum separated on an acetylated filter paper strip, Schleicher & Schüll 2043 b, and the corresponding protein diagram evaluated with Spinco's photometer Analytrol. Note the assymetrical albumin peak indicating prealbumin.

tion with hydrochloric or acetic acid, between 8.6 and 10.2) gave no addi- al separation but a broadening of the min band. Boric acid and TRIS in com- tion gave a good separation of β into two ponents and a tendency towards a split- of the albumin peak. In the same way TA with TRIS showed a tendency to- ds splitting the albumin and the β-glo- but gave at the same time two well ned α_2 peaks. According to these experi- ts a buffer of TRIS, EDTA and boric was found to be the best, giving a sepa- n of normal serum into nine fractions. ll three types of paper used in the experi- ts gave a separation into nine fractions. h cellulose acetate paper two completely ted albumin components appeared, the r migrating component being consider- smaller than the slower. With "What- man I" and "Schleicher & Schüll 2043 B" two albumin components were also recorded but the separation was incomplete. Whether the first mentioned albumin separation is due to the same components as the latter has not yet been determined.

As the cellulose acetate paper can be made transparent by oil with an appropriate refractive index, it is particularly well suited for direct photometry (Kohn, 1957). However, it is important that the strips are of good quality and particularly that the structure is homogenous.

The results of two experiments with normal serum are shown in Figs. 1 and 2. Fig. 1 shows a cellulose acetate strip stained with

Amido-Black and the corresponding diagram. Fig. 2 shows a strip of "Schleicher & Schüll 2043 B" filter paper stained in the same way. In order to determine the relation of the new fractions to those obtained with barbital buffer, the following experiment was made. A normal serum was electrophoretically separated on a starch block (Kunkel & Slater, 1952) with barbital buffer, pH 8.6, ionic strength 0.1 and the five main fractions were isolated and concentrated. The fractions thus obtained were now subjected to paper electrophoresis using the new buffer described here. The α_2-fraction was, in this way, split into two components and the β-fraction into three. No new designations of the different components in Figs. 1 and 2 have been suggested here because of the uncertainty in the nomenclature which has arisen since Smithies' (1955) observation of an even greater number of components.

DISCUSSION

It is difficult to survey the literature to determine to what extent systematic investigations have been made concerning the possibilities to separate serum proteins electrophoretically in different buffer systems, but according to Wunderly (1956), Michaelis' barbital buffer of pH 8.6 with minor modifications is unsurpassed to date. However, a greater chanse of obtaining a better separation can probably be found by proper selection of the buffer components.

Thus albumin has been divided into four components with an acetate-NaCl buffer of pH 4.0, ionic strength 0.05 (Saifer & Corey, 1955). With a borate buffer a separation has been obtained of α_2 (Hauser & Lynn, 195 and β into two components each (Consd & Powell, 1955) but at the same time separated poorly from the albumin. A di sion of β into two components has been o tained by adding calcium to barbital buff with pH 8.6 (Laurell, Laurell & Skoo 1956).

Filter paper of different quality and man facture is usually used as the supporti medium in electrophoresis. However, a ce tain amount of work has been done to fi other supporting media which could contr bute towards a better separation of the seru proteins. Good results in this respect ha been obtained with "fluid film" of agar-g (Ressler & Jacobssen, 1955; Ressler & Za 1956) and particularly with starch-g (Smithies, 1955).

The results achieved with the buffers a media described here contribute to an i proved electrophoretic separation and are interest because of the separation of the alb min into two components with an improv and more constant separation of the oth fractions. This method does not give a sep ration into so many fractions as does starc gel electrophoresis according to Smithi (1955) but as starch-gel electrophoresis not so easily applicable in routine use, i provements in paper electrophoresis shou continue to be of value. The method describ above is now used in our laboratory for d ferent purposes in investigations which a to be published later, *i. e.* the method h been found well suited for studies of prote bound serum polysaccharides after perio acid oxidation and staining with Schi reagent (Köiw & Grönwall, 1952; Björ sjö, 1955).

SUMMARY

electrophoresis buffer consisting of ydroxymethylaminomethane (TRIS), enediaminetetracetic acid (EDTA) and acid is described. In paper electrophothis buffer gives a separation of the n proteins into nine fractions: prealbualbumin, three α-components, three β-onents and γ-globulin.

ACKNOWLEDGMENT

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STUDIES ON THE DIRECTLY REACTING BILIRUBIN

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The bilirubin occurring in natural materials gives a positive van den Bergh reaction with diazotised sulfanilic acid. Due to differences in the reaction, bilirubin is termed direct if the color develops without the addition of alcohol in contradistinction to the indirect one. Various opinions concerning this difference have been presented in the literature. In 1953 Cole, Lathe & Billing, using reverse-phase chromatography with silicone-treated kieselguhr, succeeded in separating two pigments, I and II, which exhibit a direct van den Bergh reaction. This method was also applied for separation of the diazo pigments. As stated by the authors, pigment I gives rise to two diazo pigments A and B, pigment II to diazo pigment B and bilirubin to diazo pigment A. Our purpose was to obtain further information on the nature of these pigments.

MATERIALS AND METHODS

The materials used were human gallstones and bile, and urine from patients with obstructive jaundice. The method of Cole, Lathe & Billing was applied to the urine and bile in a partly modified form. After saturation of the urine and bile with $(NH_4)_2SO_4$, butanol was added up to a final concentration of 1 per cent and the solution was allowed to stand for about 10 min. After centrifugation the pigment layer was removed and dissolved in water and the solution was lyophilised. Natural bile could be lyophilised without previous purifi tion. These preparations, which were complet soluble in water, 0.1 N NaOH and Na_2CO_3, did not completely dissolve in NH_3, pyridine glacial acetic acid, were rather stable at ro temperature. The gallstones were used ground a mortar and used without purification.

To a small amount of these powders was ad 0.5 ml of water or chloroform, followed by 0.5 m freshly diazotised sulfanilic acid and 2 ml of et nol. The color develops also without ethanol, the chromatography is improved by its use. diazo reagent was prepared by adding 0.3 ml 0.5 per cent solution of $NaNO_2$ to 10 ml of per cent solution of sulfanilic acid in 0.25 N H Two methods for separating the diazo pigments paper chromatography were employed. One reverse-phase chromatography on Whatman N paper made water-repellent with Dow Corning Wacker Silicone. We used the ascending tech with butanol, 0.1 m citrate buffer and w (50 : 5 : 45) as solvent. In the other method, on Whatman No. 1, the solvent system was et acetate, pyridine and water (2 : 1 : 2).

The diazo pigments were also investigated paper electrophoresis, using the hanging method on Munktell filter paper No. 20/150. electrophoresis was carried out with barbital fer, pH 8.6, ionic strength 0.05, time 5—7 voltage 600—400 V., using five strips 4 × 35 at each run.

RESULTS AND DISCUSSION

As proved by the chromatograms, u bile and gallstones yield two diazo pigm

bilirubin (Hoffman- LaRoche and . H.) yields one diazo compound. On everse-phase chromatogram the Rf va- of urine diazo compounds were 0.2 and hose of bile 0.1 and 0.3 and that of gall- s and bilirubin 0.3. On the other hand, e solvent system ethylacetate-pyridine- the Rf values of urine and bile were 0.35 and 0.7—0.8. We obtained for gall- s not only a strong spot Rf 0.7—0.8 lso a weak spot Rf 0.3—0.35 with this od. The Rf value of the only bilirubin was 0.7—0.8. In paper electrophoresis were two fractions of urine and bile ometimes also of gallstones, while bili- gave rise only to one fraction. Under onditions mentioned above the bilirubin moved about 4 cm and the other zones cm and 4—5 cm respectively.

cording to our studies, our slower mov- pot corresponds to the diazo pigment B le, Lathe & Billing. While our investi- ns were in progress, Billing & Lathe 5) stated that pigment B is a glucuro- of bilirubin. Shortly after Schmidt 5) also reached the same conclusion studying the diazo pigments by paper natography, using as solvent system ylethylketone, propionic acid and water. stated that her diazo pigment, which sponds to our slowly moving fraction, ived from a diglucuronide of bilirubin, the other fraction, corresponding to aster moving one, is from a monoglu- ide. During our experiments it was d that in spite of the lyophilisation of reparations, the slower fraction continu- iminished. while the faster one corres- ngly increased in intensity. Using the lysis method of Schmidt (1 N HCl for 1 hr. at 100° C.) we were able to show that both of our spots when chromatographed on silicone-treated paper contained glucuronic acid. This was shown in paper chromatography with ethylacetate-pyridine-water as solvent and developed with aniline-hydrogen-phthalate. Also Sakamoto (1956) suggested that the bilirubin in dog bile is mainly in the form of ester and that the directly reacting bilirubin can be precipitated from jaundiced urine by saturation with ammonium sulphate. Other precipitating agents are acetone, according to Mertz & West (1956) and alcohol for jaundiced urine as found by us. When chromatographed by us, pigment precipitated by any of these three agents, gave rise to both diazo spots. In our investigations, bilirubin in urine and bile precipitated with ammonium sulphate did not dialyse against water in 24 hours. This suggests that there are some colloids present which prevent bilirubin from dialysing, or the glucuronic acid-bilirubin complex is linked to some larger molecule which is not dialysable. In hydrolysis of the diazo spots including the filter paper, the paper gave rise to many disturbing spots when chromatographed to demonstrate the glucuronic acid. Thus we could not definitely state whether compounds other than glucuronic acid reacting with aniline hydrogen phthalate were present. To overcome this disadvantage the pigment precipitated from urine with alcohol and dialysed was hydrolised. In addition to glucuronic acid we now obtained spots corresponding to galactose, mannose and glucosamine, when the chromatogram was developed with aniline hydrogen phthalate. When chromatography of the aqueous solution of the precipitated and dialysed pigment was carried out in ethylacetate,

pyridine and water, all the pigments remained at the starting point, but when an amount of 0.25 N HCl corresponding to that in the diazo reagent was added to the aqueous solution, a part of the pigment moved distinctly. Obviously this concentration of HCl is sufficient to split off the pigment complex. We have therefore concluded that the pigment is loosely linked to a mucoprotein through glucuronic acid.

SUMMARY

Two paper chromatographic methods are described for separating the two diazo compounds of directly reacting bilirubin from the urine of jaundiced patients and from human bile and gallstones. One is reverse-phase chromatography on silicone-treated paper, using butanol, citrate buffer and water as solvent. The solvent system of the other is ethylacetate, pyridine and water. Paper electrophoresis was also employed for separation of the diazo pigments. Both the diazo compounds contained glucuronic acid. The glucuronic acid-bilirubin complex is linked to a mucoprotein.

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QUANTITATIVE DETERMINATION OF PUTRESCINE, CADAVERINE, SPERMINE AND SPERMIDINE

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stated by Beyer & Skinner (1940), otised p-nitroaniline reacts with amines g rise to colored compounds. The color sity is related to the quantity of amine nt and allows a photometric measure-. This reaction was adapted by Beyer inner to the determination of β-phenyl-opylamine. Dessi & Franco (1949) used the same method for histamine and aline. In this paper the same method, modified, has been applied to the itative determination of putrescine, erine, spermine and spermidine. Dif-t factors affecting color formation have studied. In addition, the above men-d amines were separated by paper elec-oresis and determined with p-nitro-e.

EXPERIMENTAL

terials. The following amine salts in aqueous on were used:

putrescine bichloride,
cadaverine bichloride,
spermine tetrahydrochloride,
spermidine triphosphate.

otometric measurement was made with a -photometer using 1 cm cuvettes.

gent (Beyer & Skinner): The stock p-nitro- solution employed in forming the diazonium und was prepared by adding 3 ml of con-ted HCl to 0.69 g of the p-nitroaniline and diluting to 100 ml with distilled water. To 5 ml of the supernatant solution in a 100 ml volumetric flask submerged in an ice bath was added 1 ml of concentrated HCl. This was allowed to stand for 10 min., whereupon 3 ml of 0.7 per cent $NaNO_2$ was added and the flask put back in the ice bath for 6 min. before dilution to 100 ml with distilled ice cold water. This solution was placed in the refrigerator and allowed to stand at 0° C. for 2 hrs. before use. Unused diazonium solution should be discarded after about 18 hrs.

Technique: To 2 ml of aqueous amine solution, 5 ml of the cold diazonium solution was added and allowed to stand for 60 min. at room temperature. Then 5 ml of 1.1 per cent Na_2CO_3 was added. After 30 min. 1 ml of 10 per cent Na OH is added to develop the color. The color should be measured within 10 min. after alkalinisation.

Factors affecting color formation

Temperature. Much weaker color intensities developed at 0° C than at room temperature. At 60° C no red color appeared. Variations of 2—3° did not appreciably affect the results.

Time. In the method the reaction time is 60 min. Prolongation of this time had no effect, whereas the interval between the addition of Na_2CO_3 and that of NaOH affected the results considerably (Fig. 1). Increase of the extinction continued up to intervals of 60—90 min., at which time an intense dark color appeared in the blank. The increased color intensity afforded a possibility of making this method more sensitive. We prolonged the time from 15 min. (used by Beyer & Skinner) to 30 min. only, since the accuracy diminished and especially in the spermine test tubes a slight turbidity occurred if the time was longer.

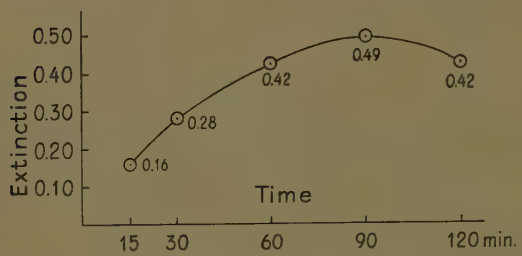

Fig. 1. Effect of the interval between addition of Na_2CO_3 and of NaOH on extinction. (Amine concentration 50 μg/ml).

Color solubility and stability. We found that the color dissolved rather well not only in n-butanol but also in isobutyl, benzyl and isoamyl alcohol, but the first mentioned proved to be the most suitable. The extraction was made directly in the test tube by adding 6 ml of n-butanol and shaking 2 min. The turbidity could not be effectively removed by centrifugation. A pinch of NaCl added before shaking and some drops of ethanol added during measurement cleared the turbidity well.

The color began to diminish in aqueous solution 10 min. after alkalinisation; in n-butanol the fading was somewhat slower.

Concentration of reagent. In order to keep the end volume small, the quantities of reagents were reduced by adding to 2 ml of amine solution only 2 ml of diazonium reagent, 2 ml of Na_2CO_3 solution and 0.4 ml of NaOH solution. It was possible to measure 2.5 μg/ml of amine salt, but the method was less accurate.

Separation of amines on paper

Based on the work of Bremner & Kenten (1951), phenol-water was used as solvent in the paper chromatography. This separated the amines but caused marked tails. The tails were not removed by impregnation with 0.2 per cent sodium acetate (Steiner & Kamenski, 1953). Butanol-acetic acid-water or variable propanol-acetic acid-water mixtures did not give better results. This paper chromatographic separation thus was not suitable for quantitative use. Since there is a theoretic possibility of separating the amines by paper electrophoresis this method was tried, with better results.

Paper electrophoresis was carried out usi citrate-HCl buffer (1/10 M disodium citrate, 1/ N HCl), pH 4.0 and 3.5 and voltage 500—450 Time of electrophoresis was 2—3 hrs. Sheets Wh No. 2 paper 46 × 20 cm were used.

Technique. A given quantity of amine soluti (0.1—0.2 ml) was pipetted on a 10 cm long starti line. On the same level, but separated from it, t same solution was pipetted on both margins the sheet. After the run the sheet was dried 60° C. The margins were cut off and the amin were localized by spraying with 0.1 per cent n hydrin in ethanol. The corresponding unspray portions in the middle of the sheet were cut and each amine was eluated with a solution c taining 5 drops of 0.1 N HCl in 100 ml of distil water. The solution was allowed to run along length of the strip as in descending paper ch matography. Exactly 1 ml of the solution was c lected, which took about 5 hrs. Solutions trea in the same way were used in the blank and control, thus eliminating the effect of the buf remaining in the paper. The amines were determi quantitatively by p-nitroaniline, as above describ reducing the quantity of reagents to one half, si the quantity of amine solutions was only 1 ml.

RESULTS AND DISCUSSION

The absorbancy peaks are in the visi range, that of spermine being at 470 n and of the others at 500 mμ in aqueous so tion. In n-butanol the peak of all the ex mined amines was at 530 mμ.

The above mentioned wavelengths w used in the determination of the calibrati values presented in Table I. As seen fr the table, the color produced by sperm and spermidine dissolves rather poorly n-butanol. The color intensity of putresc and cadaverine is increased somewhat extraction, but so little that it is of con quence only when the color is to be se rated from other colors which are not solu in n-butanol.

ble I. *Calibration values in aqueous and n-butanol solutions.*

ine lt nc. ml	Extinction							
	Putrescine		Cadaverine		Spermine		Spermidine	
	aqueous	n-but.	aqueous	n-but.	aqueous	n-but.	aqueous	n-but.
	0.04	0.04	0.04	0.05	0.04	0.03	0.04	0.03
	0.08	0.09	0.07	0.10	0.07	0.06	0.06	0.05
	0.29	0.32	0.25	0.35	0.18	0.13	0.15	0.12
	0.49	0.54	0.42	0.60	0.34	0.16	0.27	0.19
	0.74	0.80	0.59	0.87	0.53	0.23	0.45	0.25

s stated above, prolongation of the interetween the addition of Na_2CO_3 and that aOH made the method more sensitive, ling the measurement of 5 μg/ml of amialt. With lower reagent concentrations uantity of color solutions is also reduallowing the measurement of 2.5 μg/ml. ie calibration curves are not quite ght. However, in the range 5—100 μg/ is a straight line for spermine and sperie, but pucrescine and cadaverine deviate tly from the straight line. The accuracy e method is ± 10 per cent for putrescine cadaverine. With spermine and spermian accuracy of at least ± 5 per cent e reached. The preparation of the rea- requires special attention, as it must andardized as well as possible.

ie method in this form is not suitable for n paper.

paration of the amines by paper electroesis was successful at pH 3.5. In 2½ he amines moved as follows (Fig. 2):

Fig. 2. Separation of amines in paper electrophoresis. Citrate-HCl buffer, pH 3.5, voltage 500 V., time 2.5 hrs. Pu = putrescine; Ca = cadaverine; Sd = spermidine; Sp = spermine.

putrescine	21.9 cm
cadaverine	20.9 »
spermidine	19.7 »
spermine	18.2 »

For separation of spermine and spermidine pH 4.0 seems to be the most suitable.

The recovery by this method was 85 to 90 per cent.

SUMMARY

The application of p-nitroaniline to the photometric determination of putrescine, cadaverine, spermine and spermidine was studied. The method is suitable for the concentration range 5—100 μg/ml (amine salt). The accuracy of the method is ± 10 per cent for putrescine and cadaverine; at least ± 5 per cent can be attained with spermine and spermidine.

Various factors affecting the reaction are discussed. The calibration values in aqueous and n-butanol solutions are reported.

A paper electrophoretic method for separation and quantitative determination using p-nitroaniline is reported. The recovery was 85 to 90 per cent.

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POLYTHIONATE — A NEW REAGENT FOR THE DETECTION AND DETERMINATION OF PROTEIN IN URINE*)

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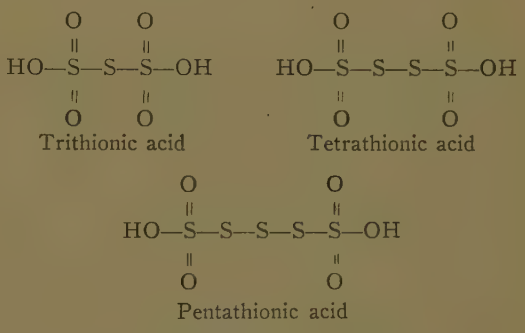
few years ago the author made the rvation that proteins are precipitated by solutions of alkali polythionates.

olythionates are salts of polythionic acids ie general formula $H_2S_nO_6$ ($n = 3$—6 higher). The tetra- and pentathionates relatively stable in acid solution, while trithionates are the most stable of the s in alkaline solution. Acidified solutions trathionate, for instance, will keep for a iderable period of time as long as the entration of the acid is relatively high. ss acid solutions the tetrathionates slowly mpose into a mixture of tri- and penta- iates, so that dilute solutions usually will ain mixtures of these salts. The decom- ion is greatly accelerated by thiosulfate, h latter is generated from polythionates eutral or alkaline solutions.

here has been much discussion about the ture of these substances, and the ques- has not been settled until quite recently ie thorough investigations of *Foss* and olleagues (Foss, 1947; Foss, Furberg & Zachariassen, 1954; Foss & Zachariassen, 1954; Foss & Tjomsland, 1955). These authors showed that the sulfur chains are unbranched although non-planar. The six oxygen atoms present in the molecule are arranged in two groups of three, surrounding the terminal sulfur atoms in approximately tetrahydral positions. The formulas of the tri-, tetra- and pentathionic acids may be written as follows:



Polythionates are weak oxidizing agents which, no doubt, will react with sulfhydryl groups in proteins. Whether the resulting denaturation of the proteins, which may be assumed to make the proteins more rigid and less soluble, together with acidification, is the only cause of the precipitation of proteins by acid polythionate solutions is not quite certain. It might also be that polythionates

*) Preliminary reports of this work were given e 8th meeting of Scandinavian Chemists, Oslo, 14—17, 1953, and at the Annual Meeting of lorwegian Society of Internal Medicine, Nov. 1954. The method is patented in Norway, en, and the United States.

combine (*e. g.* form mixed "disulfides") either with the native or the denatured proteins, and thus participate in the formation of the precipitate.

It seemed logical to find out if the new reagent could be used for the detection and determination of protein in biological fluids and, especially, if it could be used for the assay of pathological amounts of protein in urine. Qualitative tests could indeed easily be worked out, either in the form of a "ring test" to replace Heller's test (still most widely used in the Scandinavian countries), or of a "warm test" similar to Exton's sulfosalicylic acid test. None of these tests, however, represented a definite advantage over the tests in use.

When, however, the original reagent was further diluted and the volume of urine added was reduced, *e. g.* when one part of urine was added to ten parts of a 0.2 per cent polythionate solution, the test became suitable not only for the qualitative demonstration of protein in urine but also for the subsequent quantitative measurement of the amount present.

The procedure finally adopted, to be described in the following, is as easily performed and requires the same time and apparatus as a thymol turbidity test.

METHODS

1. Polythionate method.

Reagent. The reagent is an acid (pH $\leqq 2$) 0.2 per cent solution of polythionate. It may be prepared either by diluting a stable acid 5 per cent solution ("Sulfothiol") [1] or by dissolving 0.2 g potassium polythionate [1] and 0.2 g sulfosalicy acid in 100 ml distilled water.

With these two modifications of the reag practically identical results are obtained. This v shown to be the case in a series of 223 urines which parallel determinations were carried out the Clinical Laboratory of the Medical Depa ment A, Haukeland Sykehus, Bergen. In t series a correlation coefficient $r = 0.992$ was fou

On standing at room temperature, growth v usually appear in dilute polythionate solutions af some time as they seem to form good media certain moulds. It is, therefore, advisable to ma up of the dilute reagent not more than can be u up in the course of a week or so. Concentrated sta polythionate solutions, on the other hand, act fungistatics; they can be stored at room temperat almost indefinitely.

Qualitative test.

If only a qualitative demonstration of pat logical amounts of protein in the urines is atter ted, one may use as little as one or two millimet of reagent and five or ten drops of urine respe vely. The finely dispersed turbidity developing the course of a few minutes is easy to see "false" precipitations are very rare. If lar amounts of protein are present, a precipitate usu forms which can easily be redispersed by agitat the solution.

The amount of protein can be judged from intensity of the turbidity developing and may approximately measured by comparison with rium sulfate standards (Shank & Hoagland, 19 or the standards employed by Maclagan (1944) the evaluation of the thymol turbidity test. reader may here be reminded that the latter s dards had been worked out by Kingsbury, Cl Williams & Post (1927) for the turbidim evaluation of the protein content of urines.

If, on the other hand, one desires to supple the qualitative test with a quantitative determina of the amount present, one uses quantities of rea and urine suitable for the quantitative determina in the colorimeter employed, and determines by inspection which of the urines contain pro With such samples the procedure is as follows

[1] Manufactured by Norsk Sulfo A/S, Høvik, Norway. The "Sulfothiol" solution is obtained by half-neutralization with NaOH of a mixture of polythionic acids ("Wackenroder's fluid").

Quantitative determination.

the present investigation the quantitative de-
nations were carried out in an Evelyn Photo-
c Colorimeter. A red filter (660) with a
nal transmission at 660 mμ was used, and
ed water served as blind.
begin with 0.5 ml urine was added to 6 ml
nt. When the conversion factor for the com-
on of the protein content (as g per liter)
the optical densities was determined in a
of 13 samples from 7 urines in which the
n concentration also was assessed by micro-
hlization, a factor of 12.5 was found. The
volume, therefore, was raised to 0.6 ml, and
other series of 15 urines analyzed in the
manner (samples 1—7 being the same as in
revious series) a conversion factor of 9.8 was
. The figures for the protein contents given
e tables were obtained from the optical den-
determined by multiplication with 12.5 when
l and with 10 when 0.6 ml had been used.
is quite obvious that any other photometer
be used, preferably the one routinely used in
boratory for the thymol turbidity test. The
nt of reagent and (within certain limits) the
ve amount of urine to be added to give a
nient conversion factor will have to be deter-
l for each instrument. In the Clinical Labora-
Haukeland Sykehus, a Hilger Spekker Colori-
is in use.

2. Biuret method.

ntrol determinations of the protein content in
ies of 160 urines were carried out with the
method of Hiller, Greif & Beckman (1948),
ly modified to allow the use of the Evelyn
imeter. The conversion factor for this method
determined in the same two series of urines
ove, values of 6.2 and 6.6 respectively being
. The figures in the tables were obtained by
plying with 6.5 the optical densities found for
urine. The urine volumes actually used, how-
varied considerably with the protein concen-
n (from 1.0 to 10 ml).

3. Micro-Kjeldahl method.

e micro-Kjeldahl method used as method of
ence was the following: The precipitation of the protein was carried out according to the directions given by Hiller *et al.* (1948). The supernatant after centrifugation was poured off and the rest drained away by inverting the tubes on a piece of filter-paper. The precipitate was dissolved in 2 ml 0.1 N NaOH and reprecipitated with 2 ml 10 per cent trichloracetic acid. The precipitate after centrifugation was transferred to a micro-Kjeldahl flask and incinerated with 2 ml concentrated sulfuric acid to which five drops five per cent copper sulfate solution had been added. After digestion and cooling, 5 ml distilled water was added and the flask immediately connected with a Parnas-Wagner apparatus (Closs' modification), the ammonia liberated by addition of 8 ml 40 per cent NaOH was driven over and absorbed in 10 ml 2 per cent boric acid solution to which three drops of a mixed indicator (0.1 per cent bromocresyl green + 0.2 per cent methyl red (both in ethanol) 3 : 1) were added. Titration with 0.1 N HCl.

RESULTS AND DISCUSSION

Qualitative determination

All the tests called "qualitative" in this series were read in the photometer as well as by visual inspection. This was done in order to procure data that could form the basis for a quantitative method. 1567 urine samples were analyzed in this manner from March 10 to September 23, 1953. The result of the Heller test done by the technicians of the Central Laboratory, Aker Sykehus, could be traced in 1097 of these samples. The result of this comparison are presented in Table I.

The results show that, of the specimen negative for protein by the Heller test, approximately 5 per cent are positive by the polythionate method. Also, of the specimens negative by the polythionate method, approximately 5 per cent are positive by the Heller test. Determination of protein by the biuret method demonstrated that the poly-

Table I. *Comparison between qualitative polythionate test and Heller's test.*

			Polythionate		
			Positive	Trace	Negative
Heller		1097	203	40	854
	Positive	150	122	17	11
	Trace	88	30	23	35
	Negative	859	51	0	808

thionate-positive and Heller-negative urine samples did, in fact, contain protein, with the exception of 5 samples (see Table II).

False precipitations are less frequent with the polythionate method than with the Heller test. On the other hand, when using the Heller test on a very cloudy urine, it is sometimes almost impossible to see a protein precipitate. Because of the high degree of dilution in the polythionate test, even extremely cloudy urines give clear solutions when free of protein.

Quantitative determination.

160 samples were analyzed for protein with both the polythionate and the biuret methods (Tables II, III and IV). In 40 of these samples the protein content was determined by the micro-Kjeldahl technique (Tables III and IV). Finally, the protein content was assayed by the method of Esbach in 40 samples (Table V). The results of these investigations are presented in Tables II, III, and V. It is easy to see from these data t

Table II. *Protein content (as g per lit in 120 urine samples determined by the biu and the polythionate methods.*

Biuret	Poly-thionate	Biuret	Poly-thionate	Biuret	Poly thion
0	0	0.3	0.4	1.2	1.
0	0	0.3	0.4	1.2	1.
0	0	0.3	0.4	1.2	1.
0	0	0.3	0.5	1.2	1.
0	0	0.3	0.5	1.2	1.
0	0	0.3	0.6	1.3	1.
0	0	0.4	0.2	1.5	1.
0	0.1	0.4	0.4	1.6	1.
0	0.1	0.4	0.4	1.6	1.
0	0.1	0.4	0.6	1.7	3.
0	0.1	0.5	0.3	1.7	3.
0	0.2	0.5	0.5	1.9	3.
0.1	0	0.5	0.7	2.0	0.
0.1	0	0.6	0.4	2.0	1.
0.1	0.1	0.6	0.4	2.1	5.
0.1	0.1	0.6	0.5	2.2	2.
0.1	0.1	0.6	0.6	2.2	2.
0.1	0.2	0.6	0.6	2.2	2.
0.1	0.2	0.6	0.7	2.2	2.
0.1	0.2	0.7	0.7	2.2	3.
0.1	0.2	0.7	0.7	2.4	3.
0.1	0.3	0.8	0.6	2.5	2.
0.1	0.3	0.8	0.8	2.5	3.
0.1	0.3	0.8	0.8	2.5	3.
0.1	0.4	0.8	0.8	2.6	2.
0.2	0.1	0.9	0.7	2.8	3.
0.2	0.1	1.0	0.8	2.8	3.
0.2	0.2	1.0	0.8	2.9	3.
0.2	0.2	1.0	0.9	2.9	3.
0.2	0.2	1.0	1.0	3.2	4.
0.2	0.3	1.0	1.1	3.6	6.
0.2	0.3	1.1	1.0	3.8	4.
0.2	0.4	1.1	1.1	3.9	9.
0.2	0.4	1.1	1.3	4.2	4.
0.2	0.5	1.1	1.5	4.4	7.
0.2	0.5	1.1	1.5	4.9	2.
0.2	0.6	1.2	1.0	5.4	8.
0.3	0.2	1.2	1.0	7.2	8.
0.3	0.3	1.2	1.1	8.6	9.
0.3	0.3	1.2	1.1	10.6	12.

le III. *Protein content of 33 urine les (as g per liter) determined by the ahl, polythionate and biuret methods.*

jeldahl	Polythionate	Biuret
0.3	0.2	0.1
0.3	0.3*	0.4
0.4	0.2*	0.4
0.4	0.3	0
0.4	0.3	0.5
0.4	0.4*	0.7
0.5	0.4	0.1
0.6	0.4	0.3
0.7	0.2	0.2
0.8	0.4	0.4
0.8	0	1.1
0.8	0.7	0.6
0.9	0.5*	0.6
1.0	0.9	1.0
1.2	0.9	1.0
1.3	1.5	1.0
1.4	0.8	1.0
1.4	1.1	1.2 1.3
1.4	1.1*	1.3
1.5	1.0	1.1
1.5	1.2*	1.7
1.5	1.3	1.2
1.7	2.1	1.7
2.0	1.2	1.6
2.2	2.2	1.8
3.9	3.7*	3.7
4.0	3.5*	3.6
5.4	6.7	4.9
5.9	4.7	3.4
6.4	5.4	5.4
6.4	5.6	5.0
8.2	9.5	8.2
9.8	10.6	1.9

ml urine.

ost instances, the results obtained by olythionate method compare favorably those found by the biuret method or ie method of Kjeldahl. On the other the agreement between the polythionate he biuret methods was relatively poor me urine samples. Since seven of these les were from the same patient and an-

Table IV. *Protein content (as g per liter) of urine samples from 7 patients determined by the micro-Kjeldahl and biuret techniques and two modifications of the polythionate method.*

No.	Kjeldahl (gN/l) × 6.25	Polythionate 0.5 ml urine F=12.5	Polythionate 0.6 ml urine F=10	Biuret F=6.5
1	2.0 2.1	2.2 2.2	2.2	1.9 2.4
2	3.1 3.3	3.3 3.3	3.0	2.8 3.1
3	3.5 3.5	3.4 3.5	3.6	3.2 3.5
4	3.5 3.6	3.7 3.6	3.4	3.2 3.5
5	1.2 1.2	1.4 1.3	1.4	1.4 1.6
6	2.5 2.3	2.4 2.2	2.3	1.8 2.8
7	0.5	0.3	0.2	0.5

Table V. *Protein content (as pro mille and g per liter respectively) of 40 urine samples, determined by the Esbach and the polythionate methods.*

Esbach	Polythionate	Esbach	Polythionate
Trace	0.3	1¼	1.1
Trace	1.4	1¼	1.5
¼	0.2	1½	1.1
¼	0.4	1½	1.5
¼	0.4	1½	1.5
¼	0.4	1½	1.6
¼	0.4	1¾	1.1
¼	0.5	1¾	1.9
¼	0.5	2	2.7
¼	0.5	2	4.3
¼	2.0	2¾	9.3
½	0.4	2¾	9.5
½	0.7	3	2.2
¾	0.8	3	6.7
1	0.6	3	7.6
1	1.2	3	9.2
1	1.3	3	9.6
1	1.6	4	2.4
1	3.2	4	8.2
1¼	1.1	5	12.2

other four were from a second patient, there is reason to believe that sometimes the quantitative estimation is made difficult by the nature of the protein present in the urine. It is impossible to say which of the methods is the most nearly correct, since we do not know the true amount of protein present. In the biuret and the Kjeldahl methods, the protein in the urine was precipitated with trichloracetic acid. It is well known that this agent does not precipitate all the proteins; in particular, glycoproteins and certain proteins of small molecular size are left in solution.

It is obvious that agreement between the three methods is best in the medium range, while it is not so good among the lowest and the highest figures. As far as the small figures are concerned this is not the least surprising since amounts less than 0.1 g per liter are below the sensitive ranges of either of the methods. The discrepancies of the high figures are more probably due to the fact that protein contents above 2—3 g per liter more often are associated with the presence of pathological types of protein in the urines.

In this connection, it should also be stressed that we do not have any absolute measure for the protein content of any biological fluid. If we could isolate and purify the protein from such a fluid without denaturation and losses, the true amount of protein present could be inferred from the dry weight. However, this is practically impossible to accomplish with a larger number of samples.

Therefore, is has been usual to determine the amount of nitrogen in the precipitated protein and then to multiply by a factor of 6.25 to estimate the amount of protein. It is quite clear that this internationally accepted methods does not always give us the tr protein content (Closs, 1955; Nielsen, 195

When the three methods compared in th paper are taken as exampels, it can be se that the nitrogen content of urine prote will not always have a constant relationsl to the amounts of the protein present, r will the ratios between the nitrogen conte and the number of peptide linkages (biu method) or the turbidity produced by p cipitation (polythionate method) be consta It is, therefore, rather surprising that t agreement between the three methods is good as it actually is. The agreement betwe the Esbach and polythionate methods is b ter than one might have suspected, but it extremely poor in the range above 2 g l liter.

CONCLUSIONS

The polythionate method presented h may be used for the qualitative assay of p tein in urine. It is as sensitive as Helle test, and appears to be somewhat m specific.

The quantitative polythionate meth shows approximately the same degree agreement with the Kjeldahl method a the biuret method (correlation coeffici $r = 0.98$ and 0.90 respectively).

The polythionate method has the follow advantages:

1. It affords a qualitative estimation well as a quantitative determination of p tein in urine samples by one procedure with sufficient accuracy and reliability clinical purposes.

2. The amount of urine required for e determination does not exceed 1 ml.

nterfering precipitations or colorations
ry rare.
Heating is not necessary for complete
tation of the protein.
The quantitative determination requi-
le time, work and equipment.
The same amount of urine is used in
es irrespective of the protein content.

SUMMARY

ew reagent for the detection and quan-
e determination of pathological amounts
tein in the urine is described. It con-
f a dilute acid solution of an alkali
ionate (pH 1.5—2.0).
e part of urine is mixed with ten parts
gent and the turbidity produced after
lf hour is either evaluated by inspec-
qualitative test) or measured in a suit-
olorimeter (quantitative reaction).
e protein contents of urine samples as-
according to the new technique com-
avorably with those determined by the
and micro-Kjeldahl methods.

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on.

ADDENDUM

In the course of the last three to four years the present method has been introduced as routine clinical method for the quantitative determination of protein in urine at Ullevål Sykehus, Dept. Krohgstøtten, Oslo, and (by my predecessor at the Biochemical Laboratory) at the Clinical Laboratory, Med. Dept. A, Haukeland Sykehus, Bergen.

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PAPER CHROMATOGRAPHY OF PHOSPHORIC ESTERS FROM ERYTHROCYTES OF MENTAL PATIENTS DURING INSULIN COMA TREATMENT

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Insulin treatment has been used on mental patients for about 20 years (for review see Bellak, 1948) although the mechanism of this therapy is nearly unknown. Despite the fact that much information has been collected about the physiological action of this hormone (reviewed by Stadie, 1954) a general theory of its function is still lacking. Two years ago Boszormenyi-Nagy & Gerty (1955) reported a change in the "easily hydrolyzable phosphorus" from erythrocytes of mental patients after *in vitro* treatment with insulin and incubation of the hemolysate.[1] Phosphoric esters from red cells of schizophrenics had earlier been studied by among others Örström & Skaug (1950) and in non-related conditions by, for example, Prankerd (1955), Prankerd, Altman & Young (1955) and Rohdewald & Weber (1956). In view of the findings of these investigators, it was thought to be of interest to study the composition of phosphoric esters of red cells in relation to insulin treatment with the aid of paper chromatography.

This paper presents the results of an in vestigation on blood from 9 mental patie and 2 normals (blood donors). The hem lysed red cells have been deproteinized trichloracetic acid (TCA), the phospho esters have been precipitated and fract nated into two groups, barium insoluble a barium-alcohol insoluble.[2] Finally these fr tions have been analyzed by paper chron tography using one acid and one alkali system.

MATERIALS AND METHODS

Fractionation. Ganguli (1953 a), Gerlach Weber (1955) and others divided the natur occurring phosphoric esters into fractions by p cipitation with barium before the chromatograp separation. This is convenient because the num of phosphoric esters is so great that it is diffi to separate them in one- or even two-dimensio chromatograms without previous fractionat The prefractionation also removes trichlorac acid (TCA) and other constituents which m disturb the separation, concentrates the samp and simplifies the identification of spots.

The Ba-insoluble (BaI) and Ba-alcohol-insol (BaII) fractions were prepared according to Page (1949), omitting only the reprecipitation the BaI fraction. The precipitates were dissolve shaking with a cation exchange resin (Bart Savage, Hughes & Marlow, 1953) and water. first Dowex-50 in the sodium form was u

[1] We were not successful in reproducing the *in vitro* experiments with insulin reported by Boszormenyi-Nagy & Gerty (1955), partly because a detailed description of the technique was not available.

[2] In the following abbreviated as Ba I frac and Ba II fraction respectively.

samples thereby underwent alkaline hydro-
some extent. When the ion exchanger later
ed in the ammonium form (Heald, 1956)
rolysis was observed.

matographic separation. Half sheets of
aper were used in two chromatographic all-
essels designed for descending one-dimen-
hromatography. Whatman No. 54 chromato-
paper was selected because these papers
y to handle in the wet condition and become
ttle under the action of molybdate reagent.
pers were serrated on the bottom edge in
o let the solvent run off. The papers were
45 min. in 0.5 per cent disodium ethylene-
etetracetic acid at pH 8.5 and three times
illed water (Gerlach, Weber & Döring,

sphoric esters being acidic substances, should
chromatographed with a neutral solvent.
acid or alkaline solvents are to be preferred
n, Bassham, Calvin, Goodale, Haas &
, 1950).

ong the different solvent systems proposed
separation of phosphoric esters and tested
authors, the following will be mentioned:
yl acetate — acetic acid — water systems
rtimer (1952) and of Löffler & Reichl
; the methanol — ammonia — water system
durski & Axelrod (1951); the propanol —
ia — water system of Desjobert & Petek
, and the ethyl acetate — formamide —
e system of Mortimer (1952).

best results, however, were obtained with
solvent mixture (the acid solvent):

Butanol	35 ml
ichloracetic acid (Burrows, Grylls & Harrison, 1952) p. a.	2 g
hanol, absolute	10 ml
ater	55 ml

is a two-phase solvent, the water phase
used in the tank and the butanol phase in
ugh.

convenient also to use an alkaline solvent,
the esters appear in quite different order
and in alkaline solvents. As the alkaline
the following mixture of Mortimer (1952)
dopted:

Methyl cellosolve	70 ml
Methyl ethyl ketone	20 ml
Ammonium hydroxide, conc.	7 ml
Water	23 ml

It was found necessary to purify this one-phase solvent. Before the addition of ammonia the mixture of the other constituents were passed by moderate suction through a Büchner funnel, half filled with the ion exchange resin Dowex-50 in the hydrogen form and previously washed with small portions of the same mixture.

The solvents were discarded after a week because of chemical changes and evaporation from the tanks. The alkaline solvent was less changed if stored in the deep freeze. With both solvents the chromatograms were developed at room temperature for about 20 hours.

Detection of spots. The chromatograms were dried 30 min. at 45° C. After this, nucleotides and other compounds which absorb ultraviolet light were made visible by a television arrangement, described by Tiselius (1955). At first only a cobalt-nickel sulfate filter (Kasha 1948), which transmits about 50 per cent of the incident light between 240 and 320 mμ was used. Later it was observed that if a solution of Cation X (Kasha 1948) in a 1 cm quartz cuvette also was inserted, a much clearer picture was obtained. Cation X has a strong transmission peak at about 260 mμ. The dark spots visible on the television screen were outlined with a pencil on the back side of the chromatogram. Fluorescent sections were also outlined under an ultraviolet lamp.

For the detection of phosphate-containing spots the molybdate reagent of Burrows, et al. (1952) was used:

Ammonium molybdate, p. a.	5 g
Water	30 ml
Hydrochloric acid, conc., p. a.	15 ml
Perchloric acid, 70 per cent, p. a.	15 ml
Acetone,	to about 500 ml

The papers were dipped in the reagent, dried 15 min. at 45° C, and sprayed with a solution of ascorbic acid (Ganguli, 1953 b):

Ascorbic acid	0.2 g
Water	2 ml
Alcohol, absolute	200 ml

The chromatograms were left to dry at room temperature.

Table I.

Exp. No.	Sex	Age	Diagnosis	Duration of dis. circa yrs	Number of comata and praecomata	IU Insulin on day of investig.	Maximu effect that day
49	f	22	Psychosis psychogen.	¼	10	128	coma
59	m	44	Depressio mentis endogenica pers. schizoides	½	3	180	coma
61	f	24	Schizophrenia?	2	53	64	coma
63	m	23	Reactio neuroticodepressiva	½	0	240	resistent
70	f	23	Psychosis	½	14	100	praecoma
72	f	19	Reactio neuroticodepressiva pers. schizoides	3	5	84	praecoma
121	m	33	Reactio neuroticodepressiva pers. schizoides	3	0	160	resistent
123[1]					41	280	coma
90 A	m	61	Dementia praecox	23	—	—	—
90 B	m	46	Psychopathia, Schizophrenia	20	—	—	—
92 A	m	32	Normal	—	—	—	—
92 B	m	25	Normal	—	—	—	—

[1] Same as No. 59, 82 days later

Test substances.

Glucose-1-phosphate (G1P), dispotassium salt (Schwarz Lab. Inc., New York 17, N. Y.).

Glucose-6-phosphate (G6P), barium salt (Sigma Chemical Co., St. Louis 18, Mo.).

Fructose-1.6-diphosphate (F1, 6DP), magnesium salt (Mann Research Lab. Inc., New York 6, N. Y.).

3-Phosphoglyceric acid (3PGA), barium salt Nutritional Biochemicals, Cleveland 28, Ohio).

2.3-Diphosphoglyceric acid (DPGA), penta-barium salt (Bios Laboratories, Inc., New York 23, N. Y.).

$\alpha + \beta$-Glycerophosphate, disodium salt (Eastman, Rochester 3, N. Y.). Adenosine triphosphate (ATP),disodium salt (Sigma Chemical Co., St Louis 18, Mo.).

Adenosine diphosphate (ADP), (Sigma Chemical Co., St Louis 18, Mo.).

Adenosine-5′-phosphate (AMP), (Sigma Chemical Co., St Louis 18, Mo.).

Diphosphorydine nucleotide (DPN), (Schwarz Lab. Inc., New York 17, N. Y.).

Metals other than alkali metals were removed by precipitation or with ion exchange resin.

Subjects.

Pertinent data are collected in Table I. Most the patients contributing samples for experime 49—121 (Psychiatric clinic stock) presented sy ptoms of relatively short standing, referring to schizophrenic sphere. All patients were subjec to daily treatments with regular insulin. Some them (Nos. 51, 59, 63 and 121) had taken a hypn on the preceding night. Three samples of bl were taken on the same day from each: one im diately before injection of insulin (a), the sec about 4 hours later when the patient was m strongly affected (b), and the third 5 hours a the termination of the hypoglycemia (c). Two these patients (Nos. 63 and 121) were very li affected during the 4 hours between insulin inject and glucose supply. The blood sugar, measu according to Crecelius-Seifert at one hour interv during this period, fell to about 20 mg and 50 per hundred respectively.

Two patients (Nos. 90 A and 90 B) were chr schizophrenics (Ulleråker Mental Hospital) had not been treated with insulin for at least year. One (90 A) was given 3 × 25 mg of Chl promazine daily, the other had no treatment at

Table II.

Compounds	Acid solvent R_p	Acid solvent UV absorbtion	Alkaline solvent D	Alkaline solvent UV absorbtion	Alkaline solvent fluorescence	Spots emerging in
hosphate	1.0	—	8	—		5—10 min.
osphate	0.43	—	1	—		»
.....	0.35	—	13.5	—		»
.....	0.32	—	12	—		3 hours
.....	0.35	—	1.4	—		»
.....	0.80	—	10	—	faint green	»
.....	0.55	—	0	—	» »	»
contamination	0.41	—	24	—		several days
erophosphate	0.72; 0.77	—	18; 22			3 hours
.....	0.09	+	5	+		5—10 min.
.....	0.23	+	7.5	+		»
.....	0.60	+	16	+		3 hours
.....	0.09	—	25—48	—	blue	»

f investigation. Neither had taken hypnotics preceding night. The sampling was performarly in the morning, before the first meal. patient was known to have any major disease or endocrine disorder. One (No. owever, showed rather low blood sugar values (70—75 mg per hundred) on ad-

"controls" were a couple of young blood Unfortunately the conditions were not quite able for the two groups, as the normals robably had taken a morning meal before ood was collected.

Procedure.

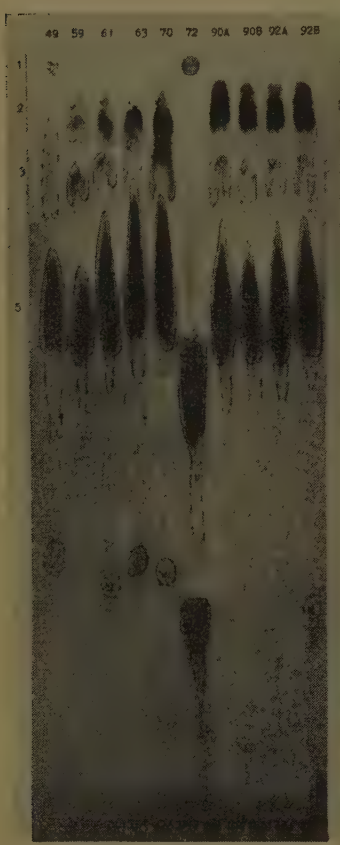
ut 50 ml of freshly drawn blood, mixed with 25 per cent heparin (Heparin forte, Vitrum, olm), were centrifuged 30 min. at 5° C. asma and buffy layers were removed. The cytes (volume V ml) were hemolyzed with nes of cold water, and 0.425 V ml 50 per /w TCA-solution was added. The precipiroteins were filtered by suction at 5° C and with 2 · V ml 5 per cent w/v TCA-solution. mbined filtrates + 0.025 · V ml 1 per cent htalein solution were neutralized with 3 H to a faint pink color, and 0.25 · V ml of cent barium acetate added. The pH was d to 8.2. The mixture was allowed to stand in the refrigerator over night and was then centrifuged.

The precipitate, representing the Ba I fraction, was mixed in a small Pyrex tube with 0.08 · V ml water and 0.04 · V g half-dry Dowex -50 ion exchange resin in the ammonium form. The tube was turned in a rotating tube holder for 1 to 2 hours in the cold room. The mixture was then centrifuged, the solution pipetted off and the resin washed with 0.04 · V ml water. The combined solutions were freeze dried in a test tube, and the remainder dissolved in 0.01 · V ml water. The solution was transferred to a small Pyrex tube, and centrifuged. Five μl of the clear solution were applied to the chromatographic paper and the remainder stored in the deep freeze.

The clear solution, after removal of the Ba I fraction, was adjusted to pH 8.2. 4 volumes of alcohol were added and the mixture allowed to stand in the refrigerator a few hours. The precipitate, Ba II fraction, was collected in a centrifuge tube, and the tube was allowed to stand uncovered over night to let most of the remaining alcohol evaporate. The Ba II fraction was then treated in the same manner as the Ba I.

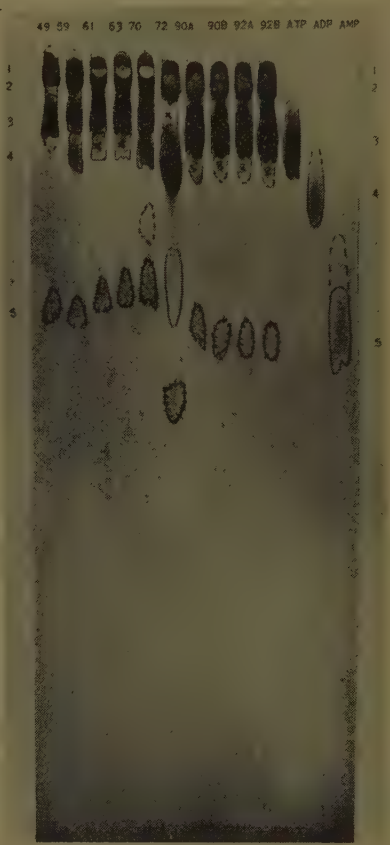
RESULTS

The chromatographic behavior of the test substances is summarized in Table II. In the



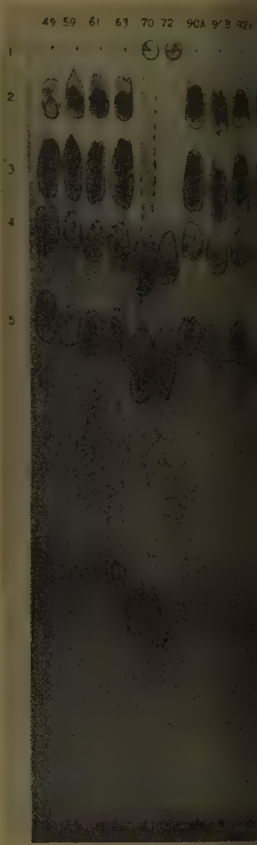
1

Fig. 1. Paper chromatography with an *acid system* of phosphoric esters from the *barium-insoluble fraction* of erythrocytes of six insulin-treated mental patients (49—72, samples obtained before the insulin injection) two chronic schizophrenics without insulin treatment (90 A and 90 B) and two normals (92 A and 92 B).



2

Fig. 2. Paper chromatography with an *alkaline system* of phosphoric esters from the *barium-insoluble fraction* of erythrocytes of six insulin-

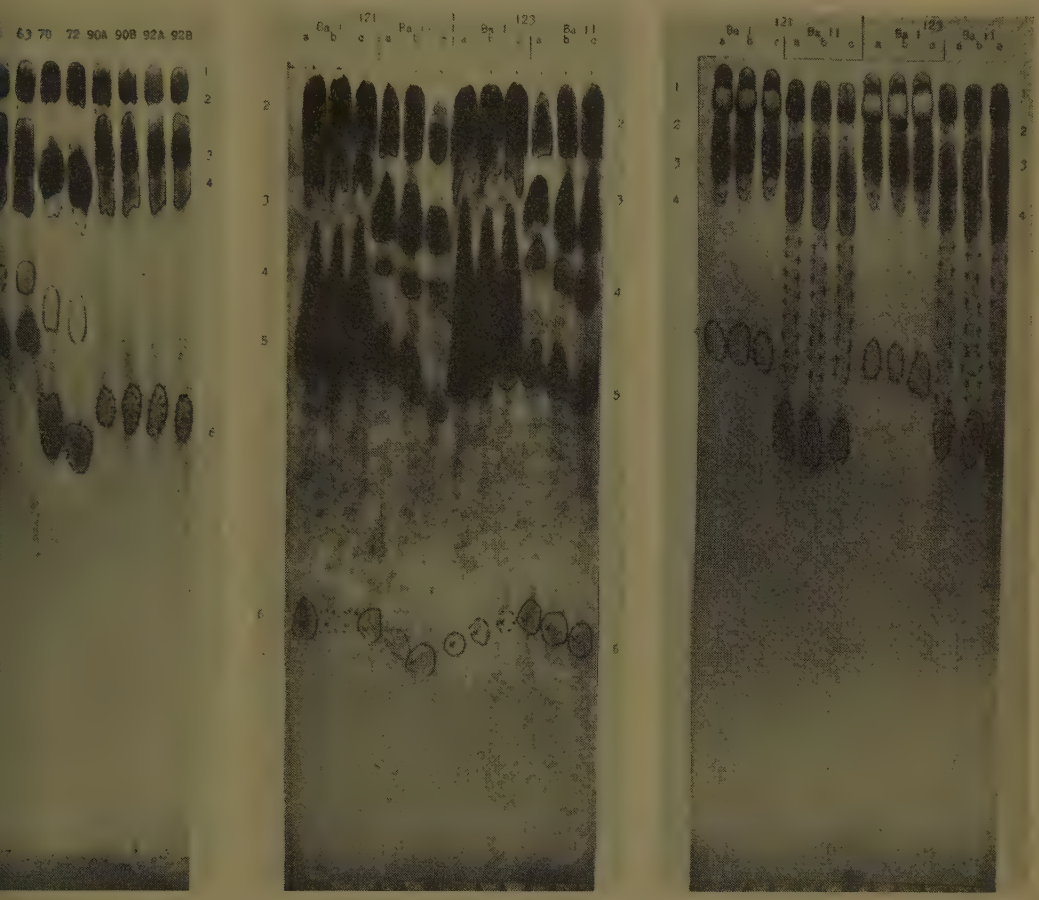


3

treated mental patients (49—72, samples obtai
before the insulin injection), two chronic schi
phrenics without insulin treatment (90 A and 90
and two normals (92 A and 92 B). On the ri
positions of ATP, ADP and AMP.

Fig. 3. Paper chromatography with an *acid sys*
of phosphoric esters from the *barium-alco*
insoluble fraction of erythrocytes. The samples
in the same order as in Fig. 1.

last column is indicated that some of the spots appeared rapidly, within 5—10 minutes after the ascorbic acid spray. These were ortho- and pyrophosphate and easily hydrolysable organic phosphates. One spot, a contami
tion in DPGA, appeared unusually slo
and did not reach full strength until a
several days. The rest of the spots were f



5

Paper chromatography with an *alkaline* of phosphoric esters from the *barium-insoluble fraction* of erythrocytes. The nples are in the same order as in Fig. 1.

Paper chromatography with an *acid system* sphoric esters from erythrocytes of two paticnts, one insulin resistent (121, the six s to the left of the midline) and one non- t (123, right half of picture). In each group the three columns on the left are samples barium-insoluble fraction, and those on the

6

right are samples of the barium-alcohol-insoluble fraction. In each group of three columns, "a" represents a sample obtained immediately before the insulin injection, "b" at the height of insulin effect (121 practically unaffected, 123 comatous) and "c" 5 hours after glucose supply.

Fig. 6. Paper chromatography with an *alkaline system* of phosphoric esters from erythrocytes. The samples and the arrangement are the same as in Fig. 5.

after three hours. The positions of the enters are given relative to orthophos- in the case of the acid solvent, and in centimeters after 20 hours development in the case of the alkaline solvent, where no suitable reference compound was at hand.

The barium-insoluble fraction (Ba I-fraction)

Fig 1 shows a chromatogram of the Ba I-fractions developed with the acid butanol-water system and Fig. 2 the same fractions when analyzed with the alkaline methyl cellosolve system. The samples are, taken from left to right, obtained from patients 49, 59, 61, 63, 70, 72, 90 A, 90 B, 92 A and 92 B. Fig. 2 also includes spots of the following reference substances: ATP, ADP and AMP (on the right side of the chromatogram). The sample from patient 72, was hydrolyzed by accident, and is the only one which shows considerable amount of orthophosphate (the fastest moving of all the spots in the acid system) as seen in Fig 1. In Fig. 1, the first two spots (levels 2 and 3) appeared rapidly, and the TV inspection had earlier indicated the presence of ultraviolet absorbing material on the same area. The color of the third spot developed fully within 3 hours and was always rather elongated. When the alkaline system was used, three spots (see Fig. 2) usually became visible in about 10 min., one in the starting position and two (touching each other) at the positions of ATP (strong color) and ADP (weak color).

The barium-alcohol-insoluble fraction (Ba II-fraction)

Fig. 3 shows a chromatogram of the Ba II-fraction developed with the acid system and Fig 4 when studied with the alkaline system. The samples are the same, and in the same order as earlier (see above). In Fig. 3 the chromatograms in the two first spots became visible in 10 min. and were localized on areas with ultraviolet absorbtion. In this series of experiments the samples fr patients 49, 70 and 72 were probably hyd lyzed, judging from the large spots of ort phosphate. In the case of the alkaline syst (Fig. 4), three spots were visible in 10 mi one in the same position as DPGA and t at levels 3 and 4, respectively, the latter co ciding with ultraviolet absorbing material

Studies during insulin treatment

In order to follow possible variations in composition of all the phosphorus compou analyzed with this technique, comparison been made of samples taken before, duri and after insulin treatment. Figs. 5 and show experiments with such samples fr two patients, one insulin resistent (No. 12 and one non-resistent (No. 123). The a system was used for the chromatogram sho in Fig. 5 and the alkaline one for the exp ment shown in Fig. 6.

DISCUSSION

Identification of the spots

The two easily hydrolysable spots wh coincide with ultraviolet absorbing mate (levels 2 and 3 in Figs. 1, 3 and 5 and lev 3 and 4 in Figs. 2, 4 and 6) are very lik nucleotides, and the comparison with the t substances indicates that they are ATP a ADP respectively. When analyzed with alkaline system, these two spots were ab equally dense in the barium-insoluble fr tion and the barium-alcohol-insoluble fr tion, whereas with the acid system ATP v stronger in the barium-insoluble fracti and ADP more pronounced in the barit alcohol-insoluble fraction. The dark el gated spot (Figs. 1 and 5, level 5) seen

rium-insoluble fraction developed with id butanol system is probably DPGA. e alkaline version of this fraction, A lingers at the starting line (Fig. 2, 1 and 2). In the barium-alcohol- in- e fraction (see Fig. 4) where the con- of DPGA is much less, the spot in 1 + 2 is likely to be due to other sub- s. Thus, the separation into fractions be helpful at least in studying such nces in the DPGA region. On the hand, the presence of about equal nts of ATP and ADP in the two frac- points to the fact that the barium- l fractionation technique gives an in- ve separation in certain respects. g unidentified spots, one seems to be tain interest, *viz.* the light spot at the ng line in the barium-insoluble fraction ped with the alkaline system (Figs. 2). A posible explanation is that this area contained acetone soluble material moved backward when the paper was d in the molybdate reagent. Disturbance phase equilibrium may cause spurious ns in connection with overlapping spots acid system.

effect of insulin. On the whole, the es of the phosphorus compounds dur- sulin treatment seem to be small. The nce of the above mentioned light spot ne starting line (Figs. 2 and 6, barium- ble fraction) is encountered only in atograms corresponding to those pati- who were treated with insulin (Nos. 2, 121 and 123) but not in the un- l cases (90 A, 90 B, 92 A and 92 B). 2 was hydrolyzed and is unsuitable for retation. Provided that this finding is nt and holds good when retested on a larger scale, the inference would be that insulin treatment causes at least one relatively durable change in the pattern of phosphorus compounds which is not visibly influenced by single injections of the hormone.

No obvious differences could be detected between the patterns of untreated schizophrenics and normals.

SUMMARY

Phosphoric acid esters from erythrocytes of 7 mental patients treated with insulin, of 2 chronically schizophrenic patients who did not receive insulin, and of 2 healthy blood donors were separated and compared visually in one-dimensional chromatograms developed with an acid and an alkaline solvent. An indication was found that insulin treatment may cause one or more substances (not yet identified) to appear, and that this substance was not influenced by single doses of the hormone. No differences were found between the chromatographic patterns of normal and schizophrenic subjects.

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CARDIAC OUTPUT IN NORMAL SUBJECTS UNDER STANDARD BASAL CONDITIONS

: REPEATABILITY OF MEASUREMENTS BY THE FICK METHOD[1]

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ht heart catheterization technique as oped by Cournand & Ranges (1941) ed the application of Fick's direct prin- for cardiac output determination ac- ng to either of the formulae:

L/min. =

$$\frac{\text{Oxygen uptake ml/min.}}{\text{Arterio-venous oxygen difference ml/L}}$$

or

L/min. =

$$\frac{\text{Carbon dioxide production ml/min.}}{\text{rterio-venous carbon dioxide difference ml/L.}}$$

ygen content is more stable than carbon le content in blood. Results are thus accurate when oxygen is used as re- ce substance (Cournand, Riley, Brad- Breed, Noble, Lauson, Gregersen & rds, 1943). Venous blood samples n from the pulmonary artery are un- onably "mixed". The potential errors nt when using right atrial samples rnand, 1945) were thus eliminated further development of the technique ed catheterization of the pulmonary artery (Cournand, Bloomfield & Lauson, 1945).

A great many investigations based on the Fick principle are recorded in the literature. Relatively few have dealt with the validity and, especially, the reliability of the method.

Even so the validity of the principle as such has clearly been proven in experiments on dogs. Thus excellent agreement in results has been obtained when the Fick method has been applied simultaneously with the direct determination of cardiac output using rotameter technique (Seely, Nerlich & Gregg, 1950).

It is generally agreed that the method is valid only if and when the subject investigated is in a steady, preferably basal metabolic, physiological state (McMichael & Sharpey-Schaefer, 1944; Cournand *et. al.*, 1945; Tanner, 1949; Cournand, Lequime & Regniers, 1951; Visscher & Johnson, 1952; Donald, Bishop, Cumming & Wade, 1953).

Several reports have been published with varying values for normal-basal cardiac output. The majority have been based on single determinations in "normal subjects", *i. e.* volunteers and/or patients without cardiovascular diease. Few have been derived from duplicate measurements in normal subjects (Cournand, 1945), or patients with essen-

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tially normal circulation (Donald *et al.*, 1953). Duplicate measurements, which define the reliability of the method by determining its repeatability (Tanner 1949), have also been performed in patients with cardiovascular disease (Harvey, Ferrer, Cathcart, Richards & Cournand, 1949).

The object of the present study has been to determine the reliability and total error of Fick's method as used at St. Erik's Hospital. Statistical analysis of duplicate and consecutive determinations in normal subjects as well as normal, resting values for cardiac output and renal blood flow are reported.

MATERIAL AND METHODS

Six female and eleven male, paid, healthy and non-hospitalized volunteers, members of the hospital staff or the Stockholm city police force were studied. Age ranged from 19 to 40 years. One subject was excluded from the original material as the catheter could not be passed into the pulmonary artery.

The studies were all carried out in the morning. The volunteers had fasted since the previous day. No premedication was given. An indwelling catheter was placed in the urinary bladder and an intravenous infusion of para-amino-hippurate (PAH) and inulin given with a constant injection syringe, was begun after the injection of a priming dose of said substances (Ek, 1955). The right heart was catheterized according to Cournand (1945). In most instances right atrial pressure was first determined and then the tip of the catheter was placed in the main stem of the pulmonary artery, where pressure was also registered. Pressures were referred to a level 5 cm below the sternal notch. An indwelling needle was placed in either brachial artery.

The urinary bladder was emptied and two to three urine collection periods, between 13 to 36 minutes long, average 21 minutes, followed. Simultaneously with the emptying of the bladder which was rinsed with water and air, arterial blood was sampled for clearance determination.

Between 34 to 79, average 57 minutes, after t catheter had been introduced, cardiac output w determined. Expired air was collected in a Tis spirometer which was rinsed three times with litres of expired air each time during a total approximately 10 minutes. Subsequently, during two minute period, expired air was collected sim taneously with the sampling of brachial and p monary arterial blood and ECG recording. Wh the voluteer continued to breathe through t mouthpiece, the spirometer was disconnected a about one litre of air was allowed to escape bef duplicate samples for analysis on Haldane's app ratus were taken. Immediately following this, t procedure was repeated. No second arterial sam was taken, however.

The duplicate air samples were analyzed accor ing to Haldane. Duplicate analyses of blood samp were performed by the same two experienc laboratory assistants according to van Slyke.

The following statistical formulae and abbr viations have been used (Dahlberg, 1940).

$$\text{Mean, } M = \frac{S\ (x)}{n}$$

$$\text{Standard deviation, } SD = \sqrt{\frac{S\ (x-\bar{x})^2}{n-1}}$$

$$\text{Standard error of the mean, } SE_M = \frac{SD}{\sqrt{n}}$$

As the study is an analysis of duplicate det minations, the differences between the two h been analyzed and the entities then deno with "d".

Standard error of a single determination (or error of the method) $SE_x = \frac{SD_d}{\sqrt{2}}$

Significance was tested by the t-test.

RESULTS

Oxygen content analysis. The results of oxy content analysis in two samples from each of two consecutively collected expired air volumes listed in Table I. In the first set of samples, difference between duplicates was more than vol. per cent only in subject 1034 (0.09 vol. cent). Owing to a technical mishap, only a si analysis could be performed in subject 976. In second set of samples the difference in subject

ble I. *Statistics on results of duplicate oxygen and carbon dioxide content analyses xpired air as well as pulmonary ventilation data from repeated measurements of cardiac output in 17 normal, healthy subjects.*

Pulmonary ventilation L/min.		Expired air CO_2 content Vol. per cent				Expired air O_2 content Vol. per cent				Respiratory quotient	
		I		II		I		II			
I	II	a	b	a	b	a	b	a	b	I	II
5.81	5.61	4.15	4.14	4.25	4.25	15.86	15.89	15.86	15.93	0.78	0.81
5.24	4.81	4.08	4.06	4.34	4.32	16.10	16.16	15.62	15.63	0.81	0.78
5.22	4.98	3.37	3.36	3.82	3.78	16.56	16.60	16.73	16.78	0.73	0.88
4.33	4.75	3.83	3.80	3.60	3.63	16.05	16.07	16.70	16.66	0.74	0.82
5.59	4.31	3.90	3.88	3.80	3.79	16.61	16.62	16.16	16.21	0.88	0.76
5.00	5.37	3.60	3.63	3.60	3.58	16.43	16.43	16.72	16.69	0.76	0.81
7.44	7.82	3.37	3.33	3.42	3.44	17.22	17.26	17.22	17.28	0.88	0.91
6.76	5.97	3.97	4.03	3.86	3.92	16.39	16.39	16.02	16.03	0.85	0.75
6.62	6.91	3.76	3.71	3.34	3.40	16.30	16.34	16.84	16.80	0.77	0.78
5.96	6.16	3.54	3.62	3.29	3.31	17.01	16.95	16.89	16.92	0.88	0.78
5.29	4.83	3.74	—	3.86	3.90	16.30	—	16.13	16.15	0.76	0.77
7.34	7.28	3.77	3.76	3.76	3.74	16.63	16.57	16.34	16.35	0.84	0.78
7.91	9.28	3.25	3.27	3.39	3.45	17.63	17.69	17.56	17.54	0.99	1.01
6.40	5.15	3.26	3.24	3.44	3.43	17.47	17.48	16.80	16.85	0.92	0.80
9.00	6.05	3.02	3.08	3.16	3.14	18.02	17.93	17.02	16.99	1.03	0.76
		14		15		14		15			
		3.64		3.67		16.74		16.58			
		-0.003		0.013		0.007		0.010			
		±0.011		±0.008		±0.012		±0.010			
		±0.041		±0.033		±0.046		±0.037			
		0.029		0.023		0.033		0.026			
		(0.79%)		(0.63%)		(0.20%)		(0.16%)			
		Difference II—I									
17		14				14				17	
5.95		3.65				16.68				0.82	
0.31		0.009				-0.122				-0.018	
±0.23		±0.059				±0.118				±0.024	
±0.96		±0.220				±0.440				±0.100	
0.68		0.156				0.311				0.071	
(11.43%)		(4.27%)				(1.87%)				(8.66%)	

07 vol. per cent, in all others 0.06 vol. per r less.

subjects 894 and 895 the air analyses were rformed by regular laboratory assistants and these results have therefore not been included in the statistics. A mean value of three analyses on each samples has been used when calculating oxygen uptake in these subjects.

Table II. *Statistics on results of duplicate oxygen content analyses in arterial and mixed venous blood in repeated measurements of cardiac output in 17 normal, healthy subjects.*

Subj.	Arterial blood oxygen content vol. per cent		Mixed venous blood oxygen content vol. per cent			
	I		I		II	
	a	b	a	b	a	b
851	17.58	17.62	12.57	12.53	12.99	12.96
855	18.18	18.01	14.89	14.93	14.79	14.85
867	14.44	14.51	11.40	11.41	11.14	11.21
886	14.07	14.03	10.95	10.93	10.97	10.92
894	15.36	15.24	11.18	11.18	11.27	11.27
895	16.41	16.50	11.50	11.47	11.96	12.11
902	15.98	15.88	12.54	12.53	12.30	12.36
907	14.78	14.57	11.67	11.72	11.77	11.69
944	17.24	17.30	13.51	13.43	13.38	13.41
964	15.71	15.68	12.59	12.64	12.27	12.26
966	17.33	17.52	13.29	13.27	13.07	13.18
972	15.27	15.27	11.84	11.89	11.58	11.77
976	17.44	17.43	13.07	13.03	12.86	12.69
986	18.51	18.45	14.63	14.68	14.49	14.54
1003	20.27	20.38	14.84	15.01	15.07	14.91
1033	16.03	16.01	13.01	13.00	12.84	12.86
1034	14.86	14.89	11.10	11.14	11.62	11.69
n	17		17		17	
M	16.43		12.63		12.62	
M_d	0.010		—0.012		—0.018	
SE_d	±0.025		±0.014		±0.024	
SD_d	±0.103		±0.057		±0.097	
SE_x	0.072 (0.44%)		0.040 (0.32%)		0.069 (0.54%)	

The difference between duplicate analyses of arterial blood oxygen content were all less than 0.20 vol. per cent except in subjects 907 where it was 0.21 vol. per cent. In the two sets of venous blood samples no difference exceeded 0.20 vol. per cent (Table II).

Ventilation and oxygen uptake. Table I l pulmonary ventilation and respiratory quoti data. These were fairly constant during the t determinations in all subjects except No. 1034 v overventilated during the first period. Ventilat was then 9.00 L/min. as compared to 6.05 L/n during the second period. This resulted in carl dioxide retention during the latter period : respiratory quotients amounting to 1.03 and (respectively.

Table III lists measured and predicted oxy uptake values. The oxygen uptake of subject 1(who overventilated during the first period, was and 224 ml/min. respectively, the difference t — 13 ml/min. as compared to a standard devia of 21 ml/min. for all the differences.

Arterio-venous oxygen difference (A-V O_2 dif The average value was 38.1 ml/L, SD 7.14 range 29.1 to 53.7 ml/L. Only a single arte sample was taken in each subject. The oxy content value of this sample was used in calculat both A-V O_2 differences (Table III).

Heart rate. Average heart rate was 71, ra 56 to 86 beats/min. (Table III). During the t periods, 85 and 86 beats/min. respectively w registered in subject 907. No other subject a heart rate exceeding 82 beats/min.

In subject 976 no heart rate was recorded dur the first output determination. During the sec period it was 69 beats/min. In subject 1033 h rate was 82 and 67 beats/min. respectively. In rest of the material, 15 subjects, no differe greater than 5 beats/min. was registered. H rate differed only from 61 to 58 beats/min. in s ject 1034 who overventilated during the first per

Cardiac output. Average cardiac output culated on mean values $\left(\frac{I + II}{2}\right)$ was 6.39 L/n SE 0.26 and SD 1.08 L/min. The range was to 8.82 L/min. (Table III).

In the male group, average cardiac output 6.44 L/min. (SD 1.30) and in the female gr 6.31 L/min. (SD 0.58).

Renal blood flow (RBF). A weighted m value for para-amino-hippurate clearance, *i. e.* r plasma flow, was calculated with regard to length of the urine collection periods. This v was corrected for a presumed 90 per cent r

Table III. *Statistics on results of duplicate measurements of cardiac output in 17 normal, healthy subjects.*

Age	Sex	BSA M^2	Heart rate beats/min.		Oxygen uptake ml/min.			A—V oxygen difference ml/L		Cardiac output L/min.	
			I	II	I	II	Pred.	I	II	I	II
19	M	1.93	70	70	280	267	266	50.5	46.2	5.54	5.78
24	M	1.83	74	74	235	240	242	31.9	32.8	7.37	7.32
21	F	1.63	80	78	214	189	201	30.7	33.0	6.97	5.73
22	F	1.56	77	77	199	187	197	31.1	31.0	6.40	6.03
25	F	1.45	70	70	218	225	183	41.2	40.3	5.29	5.58
27	M	1.83	56	56	243	204	242	49.7	44.2	4.89	4.62
26	F	1.82	71	66	227	197	219	33.9	36.0	6.70	5.47
23	F	1.63	85	86	215	214	204	29.2	28.9	7.21	7.25
32	M	1.97	66	71	248	258	258	38.0	38.7	6.53	6.67
24	M	1.94	77	74	290	282	264	30.8	34.3	9.42	8.22
34	M	2.17	63	66	291	270	291	41.5	44.0	7.01	6.14
40	F	1.76	81	79	219	235	200	34.0	35.9	6.44	6.55
35	M	1.79	—	69	232	219	234	43.9	45.4	5.28	4.70
27	M	2.14	71	71	306	325	297	38.2	40.2	8.01	8.08
36	M	1.87	68	61	240	289	242	54.0	53.4	4.44	5.41
31	M	1.85	82	67	203	198	242	30.1	31.7	6.74	6.25
40	M	1.91	61	58	237	224	243	37.6	32.2	6.30	6.96
							M—P*				
			16		17		17	17		17	
			71.1		239.2			38.1		6.39	
:			56—86		193—316			29.1—53.7		4.76—8.82	
I—I M			–1.8		–4.4		2.5	0.1		–0.22	
Range:			–15—5		–39—49		–41—39	–5.5—3.5		–1.24—0.97	
SE_d			±1.14		± 5.11		±4.77	±0.67		±0.158	
SD_d			±4.55		±21.06		±19.56	±2.75		±0.650	
SE_x			±3.22		±14.89			±1.94		±0.459	
			(15.5%)		(6.2%)			(5.10%)		(7.19%)	

-P = Differences between average $\frac{(I+II)}{2}$ and predicted oxygen uptakes.

tion of PAH. RBF was then calculated with lp of the hematocrit which ranged from 33 average 39.1 per cent. The average RBF 108 ml/min., range 794 to 1476 (Table IV).

od pressures. In 13 subjects right atrial re was recorded before the catheter tip was in the main stem of the pulmonary artery. right atrial pressure ranged from 0 to 5 mm verage 2.2 (SD 1.30).

Pulmonary arterial mean pressure covered a range between 7 and 13 mm Hg, average value 9.5 (SD 1.74). Average systolic pressure was 15.1 (SD 1.92), range 12 to 18 mm Hg, while average diastolic pressure was 5.1 (SD 1.39), range 3 to 7 mm Hg.

Average brachial arterial pressure was 119/70 mm Hg, mean 87 ± 12.9 SD.

Table IV. *Statistics on renal blood flow in 17 normal, healthy individuals.*

Subject	Sex	Renal plasma flow ml/min.	Hematocrit in per cent	Renal blood flow ml/min.	Mean cardiac output L/min.	Renal blood flow in per cent of cardiac output
851	M	856	42	1476	5.66	26.1
855	M	621	44	1109	7.35	15.0
867	F	680	35	1046	6.35	16.4
886	F	720	33	1075	6.22	17.3
894	F	567	38	915	5.44	16.8
895	M	806	38	1300	4.76	27.3
902	F	626	38	1010	6.09	16.6
907	F	567	37	900	7.23	12.4
944	M	606	38	977	6.60	14.8
964	M	727	38	1173	8.82	13.3
966	M	808	40	1347	6.58	20.5
972	F	722	37	1146	6.50	17.5
976	M	722	41	1224	4.99	24.5
986	M	771	40	1285	8.05	16.0
1003	M	413	48	794	4.93	16.1
1033	M	598	38	965	6.50	14.8
1034	M	661	40	1102	6.63	16.6
n		17	17	17	17	17
M		674.8	39.1	1108.5	6.39	17.8
SE		±26.72		±43.35	±0.263	±1.05
SD		±110.16		±178.74	±1.083	±4.32

DISCUSSION

Air oxygen content. The standard error (SE_x) of a single air oxygen content analysis was 0.033 and 0.026 vol. per cent or 0.20 per cent and 0.16 per cent respectively in the two sets of samples (Table I).

From the differences in average oxygen content between samples I and II, SE_x of expired air oxygen content was calculated to be 0.31 vol. per cent or 1.87 per cent. The analytical error is small as compared to the total error which thus is mainly due to biologic variation in the individual and inevitable shortcomings in collection and sampling technique.

Ventilation and oxygen uptake. It h been repeatedly shown that the error in ox gen uptake determination is minimized wh expired air is collected during not less th ten minutes. Shorter collection periods te to increase the error.

Stern & Ström (1957) have compared t errors inherent in different methods for ox gen uptake determination. When using t Douglas bag method which corresponds b to the one used in the present study, th found the error for a single determinati to be 6.8 ml/min. in 67 duplicate determi tions using 10 minute collection periods.

In the present study two minute collecti periods were used. Even so the volunte was connected to the spirometer duri fifteen to seventeen minutes in order to all thorough rinsing. The advantage of long collection periods had to be set against t disadvantage of making the volunteer breat through the mouthpiece even longer. It is w known that after 15 to 20 minutes of spi meter breathing, discomfort and restlessn increase rapidly in the average person.

The average difference between me $\left(\frac{I + II}{2}\right)$ oxygen uptake values and predict oxygen uptake values was 2.5 ml/mi range —41 to + 39 and SD 19.65 ml/m The difference was not significant ($P > 0.$

Average metabolic rate was + 1.4 p cent, SD 8.81 per cent, range —17 to + per cent. In two subjects metabolic rate w more than + 10 per cent, 894 (+ 21 p cent) and 972 (+ 14 per cent) (Table II Even if all the subjects appeared relax throughout the experiments it is natu that the individual reactions to the pro dure varied. A certain tenseness in subje

ıd 972 might explain the elevated meta-
ates, even if average heart rates were
gher than 70 and 80 beats/min. respec-
In order to keep the material entirely
ed, no subjects were excluded. Oxy-
ptake determined during heart cathe-
ion can not be expected to be an abso-
nd exact measure of metabolism even
rmal healthy individuals as the intro-
n of a heart catheter must by necessity
vhat disturb basality. Even so, a stable
ological state is readily obtained, as in-
d by the relatively low "error of the
d", 14.9 ml/min. or 6.2 per cent of the
value as well as the good agreement
en measured and predicted values.
arance determinations could be expec-
have an undesired effect on the "basal
ions". The comparison between the
t material and others (Table V) where
nce has not been determined shows
good agreement that this is highly im-
ble. Furthermore, the clearance values
ed check well with normal resting
published earlier among others by
(1951), a fact which lends further
rt to the assumption that the subjects
essentially "basal". Varnauskas (1955)
own that heart catheterization does not
nce clearance values.

arterio-venous oxygen difference. All
venous blood samples were drawn
thirty to sixty seconds from the pul-
y artery and in the middle of the two
e air collection periods. As the present
deals with the repeatability and not the
y of the Fick method, the moment to
nt fluctuations in blood gas content will
e discussed. It is generally agreed,
er, that during basal conditions and at ordinary pulmonary oxygen tensions, samples drawn as described here yield true and representative values for mean mixed venous oxygen content during the two minute period (Cournand, 1945; Cournand *et al.*, 1951; Donald, Bishop & Wade, 1954).

The SE of a single oxygen content analysis in mixed venous blood was 0.040 and 0.069 vol. per cent or 0.32 and 0.54 per cent of the mean values respectively in the two sets of samples. These small analytical errors have but little effect on the accuracy of cardiac output determination. The SE of a single pulmonary arterial oxygen content determination, calculated on the differences between average oxygen contents (II—I), was 0.194 vol. per cent or 1.53 per cent.

In twelve cases from the patient material in Table V, duplicate arterial samples were taken. The SE of a single determination of arterial oxygen content was 0.11 vol. per cent O_2 or 0.71 per cent of the mean. The SE of a single oxygen content analysis in arterial blood, calculated on duplicate analysis of single samples in the 17 volunteers, was 0.07 vol. per cent or 0.44 per cent of the mean. The small difference between these two standard errors indicates a high degree of stability of arterial oxygen content during duplicate and consecutive cardiac output determinations. Stability in arterial oxygen content in normals under unchanging conditions has been reported earlier (Drabkin, Schmidt, Bruner & Pennes 1944). Consequently, in order to diminish the burden on the technical staff, only single arterial samples were taken. Thus, duplicate A—V O_2 difference calculations were based on one and the same arterial oxygen content value in each subject.

Table V. *Data on the cardiac index of normal subjects. Resu*

Authors	Subject	n	Age
Cournand *et al.* 1943	«Normal subjects»	15	
Mc Michael & Sharpey-Schaefer 1944	Normal male volunteers	17	
Cournand 1945	Hospitalized «normals»	9	
—»—	Patients	23	
Cournand *et al.* 1945	Normal males	13	21—5
Stead, Warren, Merrill & Brannon 1945	Normal volunteers + patients	19	
Werkö 1947	Normals + patients	15	
Lagerlöf & Werkö 1948	Volunteers + patients no c. v. d.	13	23—3
Ebert, Borden, Wells & Wilson 1949	Male volunteers	12	30—4
Donald *et al.* 1953	Patients no c. v. d.	10	19—5
—»— 1955	Normal volunteers	16	19—5
Present material	Normal male volunteers	11	19—4
Present material	Normal female volunteers	6	21—4
—»—	Total	17	
—»—	Male patients	24	14—5
—«—	Female patients	49	16—5
—»—	Total	73	

* Absolute values. No BSA available.

Cardiac output. In order to ascertain whether methodical errors are independent of the material investigated, the results of statistical analysis of all other duplicate, consecutive cardiac output determinations performed at St. Erik's Hospital since October 1954 (73 patients) were compared to the same statistical figures representing the 17 volunteers (Table V). The diagnoses in the patient material were as follows: valvular disease 44, pericarditis 2, coarctation of the aorta 2, total block 2, hypertension 9. Furthermore, 14 cases admitted to the hospital for heart investigation but with essentially normal cardiovascular system are included. Patients with shunts due to congenital heart disease or A—V aneurysms were excluded from the material.

The absolute values for standard erro of single determinations of oxygen upta and A—V O_2 differences were slightly low in the normal group, but in per cent of t respective mean values they were almo identical in the two groups. Thus the fa that the normals were studied on an ambu tory basis does not imply that they were any way less "basal" than the hospitaliz patients at the time of, and throughout c diac output determination. The excell agreement also indicates that the patients not find the procedure any more tiring th did the healthy volunteers.

Figure 1 illustrates the decrease in card output and stroke volume which occurs age increases. This corroborates earlier fi ings (Cournand, Riley, Breed, Baldwin

licate measurements by the Fick method in three materials.

Cardiac index L/min./M² BSA			Oxygen uptake ml/min./M² BSA			A—V O_2 diff. ml/L		
	SD	SE_x	M	SD	SE_x	M	SD	SE_x
			154			45		
)			(240*)			45		
		0.14	133		6.10	45		1.5
		0.17						
	0.40		138	13.7		45	7.0	
			128			40		
			134			44		
			135			36		
	0.72		136	15.7		39	5.0	
	0.65	0.33	146		9.0	39		
	0.68		139	12.1		32	4.3	
	0.61	0.24	131	12.18	8.91	40	7.5	0.99
	0.36	0.28	130	12.08	7.69	34	4.1	1.00
	0.58	0.25	131	11.79	8.31	38	7.1	1.94
		0.28			8.11			1.38
		0.30			9.12			2.47
		0.29			8.79			2.16

rds, 1945). The decrease is even more t when the values are expressed per e meter body surface area (Fig. 2). is a concomitant fall in heart rate 3).

per cent of the first determination, the ge difference between duplicate deter- ions of cardiac output was — 2.6 per SE 2.53 per cent, SD 10.42 per cent ange — 18 per cent to + 22 per cent. gure 4, the duplicate determinations are d against each other. Harvey *et al.*) found a correlation coefficient of ($P < 0.0004$) in duplicate measure- in twenty heart patients. Neither SE_x e actual differences between determina- were given in the paper. Donald *et al.*) found a SE for a single determination amounting to 0.33 L/min./M² BSA in duplicate determinations in ten hospitalized patients without cardiovascular disease. From the results of 23 duplicate measurements published by Cournand (1945), 0.17 L/min./M² BSA in SE_x can be calculated. The original figures from a report by Selzer and Sudrann (1957) on duplicate determinations in 120 patients are not available for comparison at present.

In the present work, the SE of a single determination of cardiac index was 0.25 L/min./M² BSA or 7.15 per cent of the mean value in the volunteers and 0.29 L/min./M² BSA or 9.14 per cent in the 73 patients. The analytical errors have been shown to represent only a very small part of this error. Most of it is certainly due to biological

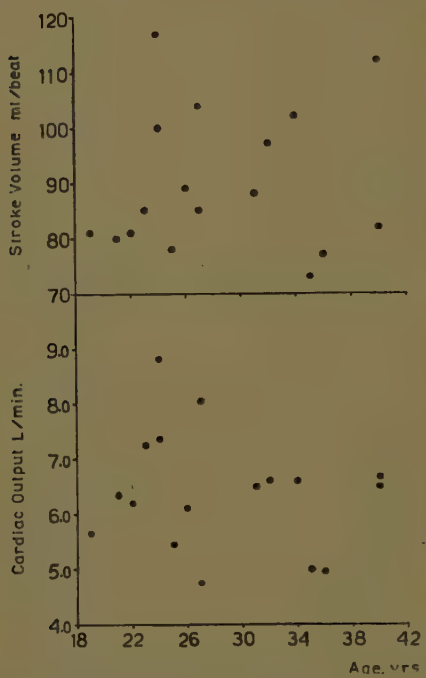

Fig. 1. The relationship between age and cardiac and stroke volumes in 17 normal, healthy individuals.

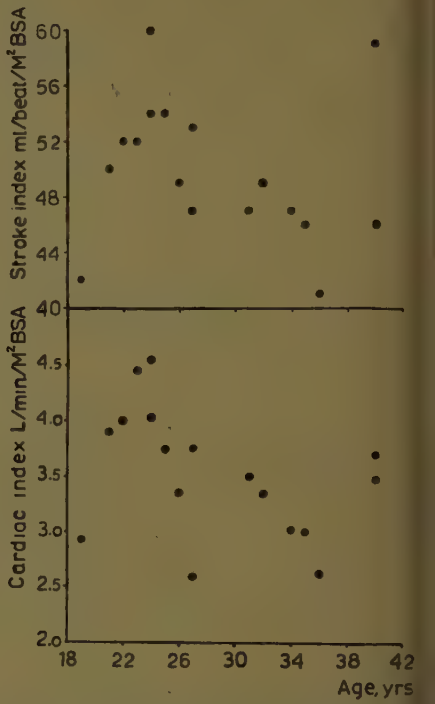

Fig. 2. The relationship between age and card and stroke indices in 17 normal, healthy individu

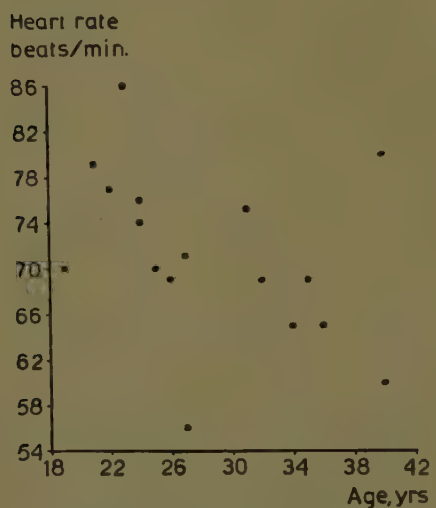

Fig. 3. The relationship between age and heart rate in 17 normal, healthy individuals.

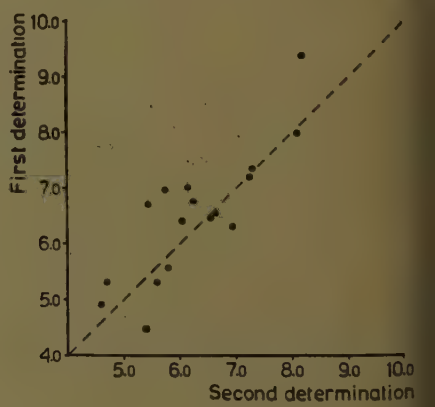

Fig. 4. Duplicate measurements of cardiac out in 17 normal, healthy subjects plotted against e other. Dotted line indicates identity between the two measurements.

on in each individual. As the accuracy iiformity of measurements, *e. g.* such pling and spirometer readings, cannot imated mathematically, no absolute for biologic variation can be given.

lly, in Table V, basal values for cardex in normals obtained elsewhere are red with the present results. Several values in the table have been obtained ysis of original figures given in the s publications mentioned. Normal cardex values ranging from 3.08 to 4.42 ./M² BSA are listed. The figure ob- in the present study, 3.52 L/min./M² seems to be a fair average of earlier .

al blood flow. The average value for blood flow per M² BSA was 608 ml/ SE 20.9, SD 83.5 and range 425 to l/min. These figures do not differ from es given in the literature. In the male RBF represented 13 to 27 per cent, ge 19 per cent and in the female group 18 per cent, average 16 per cent of c output. In the entire material the ge value was 18 per cent.

ssures. The blood pressures recorded right auricle agree with values given literature. The pulmonary arterial res, on the other hand, are definitely than what is currently regarded as ge normal (Cournand *et al.*, 1951).

SUMMARY

Duplicate and successive measurements diac output were performed in six fend eleven male healthy volunteers acg to the Fick method.

The errors in oxygen content analysis and blood were small and did not influence the accuracy of cardiac output determinations.

3. There was no statistically significant difference between measured and predicted oxygen uptake.

4. Pressures in the pulmonary artery were somewhat lower than those reported as average normal in the literature.

5. Average cardiac index was 3.3 L/min./M² BSA in male and 3.9 L/min./M² BSA in female subjects.

6. Renal blood flow represented 18 per cent of cardiac output.

7. The standard error of a single determination of cardiac output was 0.46 L/min. or 7.2 per cent of the average value.

ACKNOWLEDGEMENT

I am greatly indepted to Professor Lars Werkö and Docent Harald Eliasch for their invaluable help during the catheterization and to Docent Gunnar Ström for his kind advice on statistical problems. The renal clearance analyses were performed at the central laboratory of the hospital.

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A METHOD FOR THE DETERMINATION OF UROPEPSIN

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ıe quantity of pepsin excreted in the
: is generally considered a valuable in-
ion of the secretory activity of the sto-
ı. Several recent studies have dealt with
linical evaluation of quantitative deter-
tions of uropepsin. The determination
ɔe carried out by several methods, some
ıich are modifications of Anson's hemo-
n method (Anson, 1939). In one of
methods (Jørgensen, 1954) the quan-
of tyrosine and tryptophan liberated by
nzyme is determined by a measurement
traviolet absorption. This procedure is
enient in several respects, but the urine
be dialyzed because it contains large
tities of substances with a high absorb-
in the ultraviolet range.

ıe following is a description of a com-
ively simple and accurate method in-
ng determination of ultraviolet absorb-
Dialysis of the urine has been elimi-
l, and exact pH control has been rende-
ınnecessary.

ıe principle of the method has previously
described by the author (Christensen,
). After proteolysis, the residual pro-
substrate is determined by precipitating
trichloracetic acid (TCA), dissolving
ɔrecipitate in a urea solution, and then
uring the UV absorption of this so-
ı.

REAGENTS

Protein Substrate. For reasons discussed below, edestin is a highly suitable substrate.

The edestin employed was a commercial product manufactured by Hoffmann — la Roche. Of this protein, 2.5 g was suspended in 10 ml distilled water, and 30 ml 0.1 *M* HCl was added. In this way a clear solution of pH ca. 2.25 was obtained. To 20 ml of this stock solution was added ca. 25 ml citrate buffer (saturated with toluene) of pH 2.25 according to Sørensen. The quantity of citrate buffer was so adjusted that when 0.25 ml of the substrate solution obtained was diluted with 5 ml citrate buffer and precipitated by TCA, and the precipitate was dissolved in urea, the resulting solution had an absorbancy at 280 mμ of 1.00. Stored at 4° C. the edestin solution is stable for several months.

Citrate buffers for dilution of urine.
a. Sørensens citrate buffer pH 1.65.
b. Sørensens citrate buffer pH 1.80.
c. Sørensens citrate buffer pH 2.10.

Trichloracetic acid (TCA) (E. Merck, Darmstadt).
Ten per cent (W/V) solution (0.61 *M*).
Five per cent solution for washing precipitates.

Urea solution.
A quantity of 350 g urea (C. P.) was dissolved in 1/15 *M* Sørensen's phosphate buffer of pH 7 to make one liter.

Pepsin used for standardization was a crystalline product from Armour & Co., Chicago, prepared by Northrop's alcohol method. The prepara-

tion employed contained 93 per cent dry matter. For establishment of reference curves it was dissolved in citrate buffer.

PROCEDURE

With a Carlsberg pipette, 0.25 ml edestin substrate solution was transferred to a centrifuge tube having a circular mark at 5 ml. Next 5 ml diluted urine (see below) or standard pepsin solution was added; it had a pH of ca. 2.25. Digestion proceeded for two hours at 30° C. For determination of very small quantities of pepsin, it is convenient to extend the period of digestion to twenty hours.

After completion of the digestion period, the residual substrate is precipitated by addition of 5 ml 10 per cent TCA. About thirty minutes later the sample is centrifuged; the supernatant is easily decanted from the pellet of precipitated protein. The precipitate is washed with 5 per cent TCA and dissolved by addition of 35 per cent urea solution to the 5 ml mark. Solution is accelerated and the solution is made uniform by stirring with a glass rod. (If the tube is turned upside down after insertion of a cork or rubber stopper, an error is introduced because cork and rubber give of substances that absorb ultraviolet light).

The absorbancy of the solution at 280 mμ is determined against a 35 per cent urea blank. The values given in Tables I and II were determined by means of a "Unicam" spectrophotometer with 1 cm cells and a slit width of 0.4 mm. A standard was run in the same way with the one exception that the trichloracetic acid was added at the same time as the diluted urine. The difference between the absorbancies of the test solution and the standard can be used for determining the pepsin content with the aid of a reference curve constructed from runs with known quantities of pepsin.

The procedure may be modified in several ways, depending mainly on the extent of urine dilution.

A. The urine is diluted 1 : 5 (4 ml urine diluted to 20 ml with citrate buffer *a*) or, in the case of somewhat more concentrated, dark urine, 1 : 10, (2 ml urine diluted to 20 ml with buffer *b*). Five ml of urine of either dilution is used for digestion for a period of two hours. In this case a standard must be run for each urine sample because a small quantity of substances absorbing in the ultraviolet range will be adsorbed to the protein precipi requiring individual correction. The result is from reference curve A.

B. The urine is diluted 1 : 50 (0.2 ml is dil to 10 ml with buffer *c*). Of this diluted u 5 ml is incubated for twenty hours with the ed substrate. In this case a common standard (0.2 edestin + 5 ml buffer *c*) may be used for samples. This method is particularly conveni it is preferable when large numbers of samples examined. The result is read from refer curve B.

COMMENTS ON THE PROCEDURE

When the above buffers are used, the of the diluted urine will usually fall in range from 2.00 to 2.35. More accurate adjustment is unnecessary since the rat hydrolysis of edestin by pepsin is practic independent of pH in the region between and 2.5 (Table I).

Table I. *The pH dependence of hydrol of edestin by pepsin (digestion for two h at 30° C., 4.65 μg pepsin per 5.25 m reaction mixture).*

pH	$(E^{1\,cm}_{280})$ standard — $(E^{1\,cm}_{280})$sa
1.50	0.347
1.77	0.346
2.00	0.341
2.25	0.348
2.53	0.351

The present method, which involves termination of residual substrate, necessit the use of comparatively small substrate centrations. Consequently, proportion between the decrease in the substrate and enzyme concentration is not to be expe The reference curves of Figures 1 and 2 been drawn on the basis of the values li in Table II.

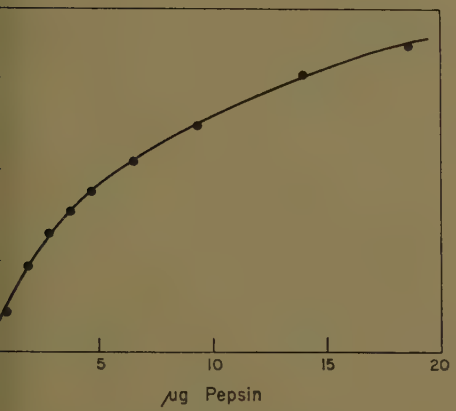

Fig. 1. Reference curve A (2 hours digestion period).

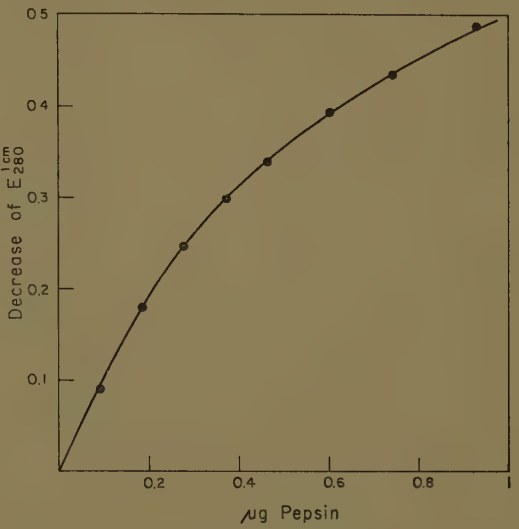

Fig. 2. Reference curve B (20 hours digestion period).

Table II.

eference curve A		Reference curve B	
psin μg	ΔE	Pepsin μg	ΔE
93	0.087	0.093	0.090
86	0.188	0.186	0.180
79	0.260	0.279	0.247
72	0.308	0.372	0.299
65	0.350	0.465	0.339
51	0.419	0.605	0.394
30	0.496	0.744	0.435
95	0.608	0.930	0.488
50	0.670		

SUMMARY

description is given of a method for titative estimation of pepsin in urine. method is based on determination of the tity of edestin substrate remaining after proteolysis with a urine sample for a given period of time. The determination is carried out by ultraviolet photometry after precipitation of the residual protein with trichloracetic acid and dissolution in strong urea solution.

The method requires neither dialysis nor pH adjustment of the urine; consequently it is suitable in cases where there are many urine samples to be examined daily.

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TITRIMETRIC ASSAY OF PROTEOLYTIC ACTIVITY

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Determination of the proteolytic activity of a given sample may often be carried out advantageously by means of a titrimetric procedure. This yields an expression of the number of peptide bonds hydrolyzed, which is often more informative than results obtained by the precipitation methods in common use. In determination of peptidase action, titration procedures may be even indispensable.

The most frequently used titrimetric methods are the alcohol titration of Willstätter & Waldschmidt-Leitz (1921) and the acetone titration of Linderstrøm-Lang (1927).

In the former, NH_3 -groups are titrated in accordance with the scheme:

$$NH_3^+\text{-R-COO}^- + OH^- \rightleftharpoons NH_2\text{-R-COO}^- + H_2O$$

The principle underlying the method is that addition of alcohol alters the dielectric constant of the medium so that the equilibrium constant for the above process and also the equilibrium constant for the dissociation of the indicator (thymolphthalein) assume values that enable the former process to go to completion before dissociation of the indicator acid sets in, which in turn occurs at a reasonable hydroxyl ion concentration. Thus the quantity of hydroxyl i needed to bring the medium itself to required alkalinity does not exceed the qu tity consumed in the reaction.

As it is usually carried out, the Willstä titration has certain drawbacks, which h probably discouraged its use:

a) Since the titration is carried t strongly alkaline reaction, there is a co derable risk of absorption of CO_2 from atmosphere and from expired air.

b) Consequently the titration must completed rapidly. This is particularly fortunate in the present case where it is sufficient to observe a color change, where the titration must be carried to a finite color intensity.

c) For this same reason it may be d cult to utilize the method in experim where samples are withdrawn at brief in vals.

d) The protein often tends to precipi which may make titration difficult and crease the obtainable accuracy.

These difficulties may be overcome by following simple procedure.

The titration is carried out in a n closed system. The titration vessels empl

ordinary penicillin bottles with remov- flat rubber stoppers. By means of rub- tubing and a short glass tube the burette onnected to a fine hypodermic needle ch penetrates the rubber cap during the tion. The hypodermic needle is fixed by ns of "De Khotinsky Cement" to a tape- piece of glass tubing that fits into the ve of the needle. It is important that the dle must go easily through the rubber per; if the latter is new it is advisable make several perforations in it with a rse needle before using it. The stopper uld fit so that the bottle can hang from needle connected to the burette, without port.

Overpressure inside the bottle is avoided neans of another hypodermic needle pene- ing the cap. The latter needle must be rse as it is used for introducing the alco- This is done with a Record syringe of r 10 ml. The alcohol is added in small tions, each time the air displaced is drawn the syringe.

A determination was usually carried out he following way: First it is ascertained much alcoholic potassium hydroxide is uired to make a sample with added in- ator so alkaline that it turns blue. This mple is obtained by withdrawal of suitable uots from the substrate and enzyme solu- s before mixing. From the burette with hypodermic needle the determined quan- is added through the rubber cap to the ation vessel containing already measured ntities of indicator and of alcohol. These ntities are so chosen that the total volume lcohol is approximately twice that of the mple. Under these conditions (ca. 66 per t alcohol and strongly alkaline reaction) the enzyme activity is assumed to cease im- mediately. To the receiver thus prepared the sample is added, the rubber cap being lifted a little. After shaking, the contents of the titration flask are generally perfectly clear. They should have a distinct blue color.

Samples are pipetted most easily by means of a constriction pipette (Carlsberg pipette). Naturally it will not do to empty it the usual way, *viz.* by blowing with the mouth. Instead it is provided with a perforated rubber bulb permitting rapid and convenient filling and emptying of the pipette. In this way a volume of 1 to 2 ml can be pipetted to within 0.1 per cent of the mean value. Consequently this method may be used even in cases where the blank is considerable (*e. g.* for deter- mination of peptic hydrolysis in strongly acid solution).

If samples of 1 to 2 ml are withdrawn from a reaction mixture containing 1 to 2 per cent protein, it will be convenient to ti- trate with 0.05 M alcoholic KOH from a 2 ml burette graduated to hundredths of a ml, the third decimal being estimated.

Under favorable conditions the obtainable reproducibility is 0.004 ml 0.05 M KOH (1 drop).

The quantity of CO_2 entering the titration vessel may be regarded as constant from one pipetting to another, and further absorption of CO_2 is excluded by the precautions de- scribed. Consequently the titration may be carried out slowly and carefully, and it need not be done immediately after sampling. Since the sample is made alkaline at once, the protein as a rule will not tend to preci- pitate. If the substrate is casein, the titration medium will remain practically water clear. It is a further advantage that the accuracy

of pipetting may be checked by weighing the vessel. This may be desirable if the substrate is viscous. In the latter case errors may arise because the volume delivered by the pipette increases as hydrolysis of the substrate causes its viscosity to decrease.

SUMMARY

The author describes a procedure by which the accuracy of the alcohol titration of Willstätter & Waldschmidt-Leitz may be creased.

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IS VITAMIN B_{12} IN BOUND OR FREE FORM IN THE TAPEWORM DIPHYLLOBOTHRIUM LATUM?

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n nature vitamin B_{12} seems to occur stly in complex-bound form. B_{12}-binding stances and B_{12}-conjugates have been nd in a large number of organisms and ues. It therefore seems reasonable to ume that in *Diphyllobothrium latum* vita- B_{12} would also occur in bound form. wever, a previous investigation by Kai- nen & Wallén (1954) indicated that vita- B_{12} in *D. latum* occurred in the free m. Since the general view is that "in its ctional forms "B_{12}" is incorporated in h molecular complexes having enzymatic perties or acting as enzyme precursors" ord & Hutner, 1955), the present authors ertained some doubts as to the correctness this observation and thought it desirable reconsider the question of whether the in *D. latum* occurred in bound or free m. The results of such an investigation reported in this paper.

MATERIAL AND METHODS

Since *D. latum* readily absorbs radioactive B_{12} inistered orally to its host (Nyberg, 1956), in present study a radioactive tracer technique used. Tapeworm carriers were given daily –1.0 μg $Co^{60}B_{12}$ (Merck) by mouth for 5—7 s, after which time the worm was expelled with *ractum filicis*. The worm was washed carefully with water and was then stored in a deep-freeze until used for this study. Care was taken to pro cess the worm quickly to prevent autolysis and action of microorganisms.

After thawing, the worm was homogenized in an equal volume of ice-cold physiological saline and the homogenate was filtered through gauze to remove larger particles.

Nondialyzability of the radioactivity was taken as the criterion of bound B_{12}. 2—5 ml of the homogenate were pipetted into thin dialysis bags (Visking Co., Nojax Casing, size 8/32), and the radioactivity of the bags was counted in a scintillation counter before and after dialysis. The bags were counted lying all in the same geometrical position in tin-foil cups. The dialysis was performed against three changes of 1000 ml each of outside liquid; the dialysis vessel was shaken at irregular intervals to speed up the diffusion of the free B_{12}. In different experiments the dialysis was performed at different temperatures. When the dialyzability of the radioactivity of the homogenate was studied, the control bags contained radioactive B_{12} and physiological saline, and the dialysis was performed against physiological saline. When the effect of various enzymes on the dialyzability was studied, enzyme solution was pipetted into some bags, and into the control bags was added an equal volume of liquid containing no enzyme. The bags were then dialyzed at 37° or 45° C. for 5 hours, after which time the dialysis was continued at 4° C. As outside liquid a buffer was used with the pH optimum of the enzyme (pH 5.5—8.0 phosphate, pH 1.9 HCl). The digestion thus took place simultaneously with the dialysis.

To check whether the results obtained with radioactive B_{12} would hold true also for the non-

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Table I. *Dialyzability of radiovitamin B_{12} absorbed by Diphyllobothrium latum. Dialysis against physiological saline. Values means of double determinations.*

Sample	CPM				
	Before dialysis	After dialysis			
		hours of dialysis			
		24	48	72	144
a. *Dialysis at 4° C.*					
Fresh homogenate with NaCN	669			678	629
without NaCN	708			732	706
Radiovitamin B_{12} (control)	876	103	0	0	0
b. *Dialysis at 37° C*.*					
Fresh homogenate with NaCN	630	196	83	0	0
without NaCN	690	230	99	0	0
Radiovitamin B_{12} (control)	738	49	0	0	0
c. *Dialysis of dry worm*					
i. Dialysis at 4° C.					
Dry worm	3008		2072	1766	
Radiovitamin B_{12} (control)	3203		0	0	
ii. Dialysis at 37° C*.					
Dry worm	3500		514	0	
Radiovitamin B_{12} (control)	2694		0	0	
d. *Effect of boiling*					
Dialysis at 4° C.					
Fresh homogenate	1900			1407	
Homogenate boiled 10 min.	1936			820	

* Toluene added to prevent growth of microorganisms.

radioactive B_{12} normally contained in the worm, some experiments were performed with worms from hosts who had not received any radioactive vitamin B_{12}. The worm was treated in a way a logous to that described above. The vitamin activity dialyzing out from bags containing hom genized worm was assayed microbiologically fr the outside liquid. The organism used was *Lac bacillus leichmannii* 313 (ATCC 7830) and assay method was the one described in Supp ment 1, 1955 of the Swedish Pharmacopea.

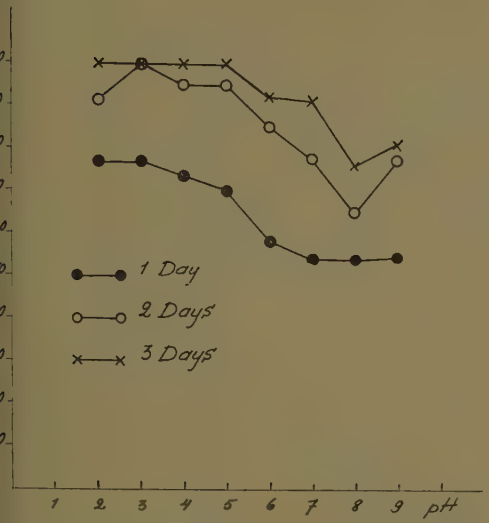
RESULTS

Nature of the radioactive substance. T worm was treated in the same manner liver in the preparation of commercial liv extracts (papain digestion, see Nybe 1952). The concentrate was finally extract with phenol-water. The evaporated extr was chromatographed in a) butanol-wa and b) lutidine-water. The radioactivity w found to have the same R_f as known sp of vitamin B_{12}. It can be concluded that *radioactive substance is a cobalamin.*

Dialyzability. Whereas vitamin B_{12} in control bags dialyzed out completely in hours, the radioactivity of the homogen did not diminish much in 144 hours wh the dialysis was performed at 4° C. Addit of cyanide to the outside liquid did not lib ate the radioactivity (Table I, experime a & b). It is concluded that *the radiovita is in bound form.*

Autodigestion and drying. When the p ceding experiment was performed at 37° the radioactivity of the homogenate dialy out completely in 72 hours (Table I, exp ment b). Evidently, *autolysis liberated vitamin.*

The effect of drying was studied in following manner: The worm was spread on tin-foil and was allowed to dry at ro temperature. After about 24 hours it co



. 1. The effect of pH on the autolytic liberation bound radiovitamin B_{12}. Less radioactivity is liberated at high pH.

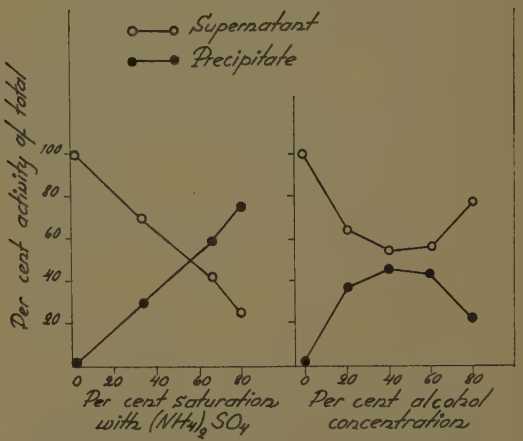


Fig. 2. The effect of ammonium sulfate and alcohol precipitation on bound B_{12}. More radioactivity is precipitated with increasing ammonium sulfate concentrations, whereas higher alcohol concentrations liberate bound radiovitamin.

powdered by grinding it in a coffee-mill. e powder was suspended in physiological ine. At 4° C. about 50 per cent of the lioactivity dialyzed out in 72 hours, where- at 37° C. all the radioactivity came out the same time. *Drying,* evidently because brings about autolysis, *thus liberates the lioactivity* (Table I, experiment c).

pH optimum of autodigestion. Dialysis at ° C. of the worm homogenate was per- med at different pH's. As is apparent from . 1, definitely less radioactivity dialyzed at high pH, whereas at low pH the radio- ivity dialyzed out quickly.

Enzyme digestion. As is seen from Table II teolytic enzymes speed up the liberation the radioactivity. There is no significant ct of DN ase and RN ase. This would icate that *the vitamin is bound to a tein.*

Precipitation. To study whether protein precipitants would precipitate the radioactivity of worm homogenate, alcohol and ammonium sulfate precipitations were performed. As is apparent from Fig. 2, ammonium sulfate precipitated the radioactivity. On the other hand, low alcohol concentrations precipitated a certain portion of the radioactivity, whereas at higher concentrations the radioactivity of the supernatant increased. Alcohol therefore seems to liberate the vitamin from the complexing substance.

Boiling. When the dialysis rate of boiled homogenate was compared to the rate of nonheated homogenate it was found that more radioactivity was dialyzable in the boiled than in the nonboiled sample (Table I, experiment d). *Boiling thus liberates the bound vitamin.*

Table II. *Effect of enzyme digestion on the dialyzability of radiovitamin B_{12} absorbed by Diphyllobothrium latum.*

Sample	CPM (mean of double determinations)	
	Before digestion	After digestion 5 hrs. and dialysis 3 days at 4° C.
Homogenate + pepsin	5180	1898
Homogenate + liquid digested at pH 1.9 and 37° C.	5343	2943
Homogenate + trypsin	4815	3167
Homogenate + liquid digested at pH 8.0 and 37° C.	4860	4218
Homogenate + papain	5553	2308
Homogenate + liquid digested at pH 5.5 and 45° C.	5632	3113
Homogen te + DN ase	7284	6450
Homogenate + RN ase	7380	6610
Homogenate + liquid digested at pH 7.0 and 37° C.	7064	6394

Table III. *The effect of different te peratures on the dialyzability of microb logical vitamin B_{12} activity in Diphyl bothrium latum. N. B. Toluene added sample and outside liquid to prevent grow of microorganisms.*

Sample	Microbiological vitamin B_{12} activity in equal amoun of outside liquid. Mean multiple determinations. μμg/ml	
	Experiment 1*	Experimen
Worm homogenate dialyzed at 4° C. for 3 days	4550	237
Worm homogenate dialyzed at 37° C. for 3 days	10525	1330

* Due to lack of suitable experimental material, first experiment was performed with a worm wh was expelled as late as 24 hours after the administ tion of the anthelminthic. It is therefore highly p bable that it was partly autolyzed.

Microbiological study on nonradioactive worm. The same amount of worm homogenate was dialyzed at 37° C. and 4° C. against equal volumes of physiological saline. Toluene was added to all inside and outside liquids to prevent bacterial growth. After 72 hours of dialysis the microbiological B_{12} activity of the outside liquids was determined. Definitely more B_{12} activity was found in the outside liquid of bags dialyzed at 37° C. This experiment supports the above conclusion that *vitamin B_{12} in D. latum occurs in bound form and that autolysis liberates the vitamin* (Table III).

DISCUSSION

The results indicate fairly conclusiv that vitamin B_{12} in *D. latum* occurs bound form, and that the binding substa is large molecular and probably a prote This seems to apply as well to (radio-)vi min B_{12} recently absorbed by the worm to the vitamin normally contained in worm.

Also *in vivo* experiments support the vi that exogenous vitamin B_{12} gets bound the worm: When inorganic radiocob ($Co^{60}Cl_2$) was administered by mouth worm carriers the worm did not absorb

ificant amounts of the radioactivity. On other hand, when radiovitamin B_{12} was en the worm was found to concentrate the min in itself; expelled worms contained the average 1.8 times more radioactivity n the stools of the host. Further, the up- e of B_{12} by the worm was greatest in its ximal third, where the growth of the pro- tides occurs (Nyberg, 1956). All these lings argue in favor of the view that the ake of B_{12} by the worm is specifically abolic. To what protein in the worm the min is bound and what role the vitamin rs in the metabolism of the parasite re- ns to be investigated.

The apparently erroneous conclusion that min B_{12} ocurred in the free form in *latum* is perhaps explained by the fact the investigators used dried worm in r experiments. Our experiment indicate drying, probably involving autolysis, rates the bound vitamin.

t is also possible that the vitamin con- ed in the worm, though bound, is directly ilable to the organism used in the micro- ogical assay, as has been found to be the e with the vitamin contained in human ues (Ross & Mollin, 1957).

This study adds further evidence in favor he view (Gräsbeck, 1956) that unreliable ılts are obtained when vitamin B_{12} bind- is studied by means of the microbial wth inhibition technique.

SUMMARY

t was demonstrated that vitamin B_{12} en up by *D. latum* is still a cobalamin, that the vitamin occurs in bound form, and that the large molecular binding substance is a protein. The binding substance — vitamin B_{12} complex can be dissociated with heat, alcohol or proteolytic enzymes, or by allowing the worm to undergo autolysis. Drying likewise liberates the vitamin. The bound vitamin can be precipitated with ammonium sulfate. The results indicate that the uptake of vitamin B_{12} by *D. latum* is specifically metabolic.

ACKNOWLEDGEMENT

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SYNOVIAL FLUID POTASSIUM

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In the pilot investigation (Kulonen & Mäkinen, 1955) we reported values of synovial fluid potassium which seemed to be of clinical interest and connected with the action of hydrocortisone. This study is now extended. The problems to be inquired into were: (1) how the potassium level is connected with the severity of the disease, (2) does the potassium level have any prognostic significance in steroid therapy and (3) whether other qualities of the synovial fluid were correlated with the potassium level?

MATERIAL AND METHODS

The patients were those from the Rheumatism Foundation Hospital, who had knee effusions large enough. When the first sample of 10 ml was drawn, 25—50 mg of hydrocortisone (Merck's Hydrocortone Acetate suspension) was injected and after 24 hours the synovial cavity was emptied. Some patients did not have enough fluid for the second sample and they are not included here. No other selections were made. The samples did not contain blood or pus.

Potassium was determined with a flame photometer using internal lithium standard. The colloid osmotic pressure was determined by a modified method of Autio (1954), the viscosity with Ubbelohde's viscosimeter, the proteins using biuret-reaction and hyaluronate calculated from non-protein glucosamine determined according to Blix (1948). The analytical details are described in the previous paper (Kulonen & Mäkinen, 1955).

RESULTS

There was a statistical, if not practicall very useful, correlation between the erythr cyte sedimentation rate and synovial flui potassium (Fig. 1). A similar relation wa found in the distribution of the cases int various potassium level groups when classe I + II and class III were compared (Fig. 2 No differences were found between th stages. In conclusion it may be stated tha high potassium levels are connected wit

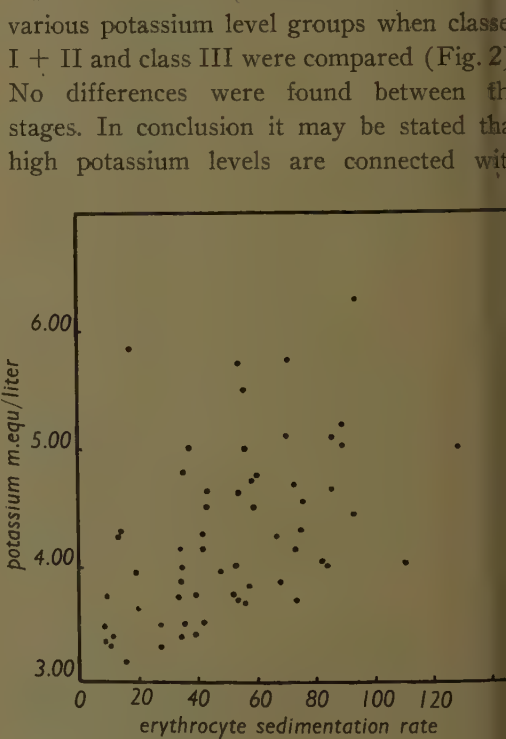
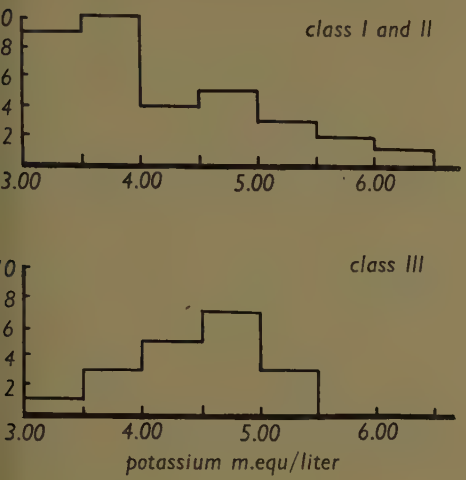


Fig. 1. The relation of synovial fluid potassiu and erythrocyte sedimentation rate. r = + 0. P = 0.002.



2. The distribution of cases of different classes ccording to synovial fluid potassium level.

e active disease but the erythrocyte sedi- tation rate reflects the activity similarly. he cases are divided into two groups: ses I + II and class III respectively, the erences in the synovial fluid potassium ls are significant ($P = 0.02$), but in the hrocyte sedimentation rates still more ortant ($P < 0.001$).

able I presents the drop in potassium l and its relation to the clinical effect of rocortisone. It seems justified to ask (1) ther this means that hydrocortisone treat- t has better chances in those cases where initial value is high or (2) whether per- s after excellent effect the final value is Analysis of the cases shows that none hese assumptions is correct and the hy- cortisone effect shows no correlation er with the initial or the final values.

Ve correlated the potassium levels with r chemical qualities of synovial fluid and nd the following correlations:

ntrinsic viscosity $r = -0.58$, $P = 0.001$
olloid osmotic pressure $r = +0.32$,
$= 0.10$.

Table I. *Changes of synovial fluid potassium levels during hydrocortisone therapy.*

Clinical effect	No. of cases	Mean change m. eq./liter	P of the chance occurrence of the difference
Poor	12	−0.13	> .10
Moderate	13	−0.27	= .10
Excellent	29	−0.53	< .01
All cases	54	−0.38	< .01

There was no correlation to the sodium or chloride levels, size of the hydrops or protein or hyaluronic acid contents.

DISCUSSION

The increased potassium concentration in the synovial fluid might be due to the irritation and destruction of the synovial cells. The amount of cells, some of them dying, is also increased in pathological synovial fluid. It is interesting that the healing effect of hydrocortisone is more connected with the absolute drop of potassium, not with the values themselves. We would like to interpret this to mean that hydrocortisone activates some metabolic change in the synovial cells where potassium is necessary.

SUMMARY

Synovial fluid potassium is closely related to the activity of the disease and correlated to other characteristics (erythrocyte sedimentation rate, "class", intrinsic viscosity, colloid osmotic pressure), which also reflect the activity of the rheumatic process.

Hydrocortisone decreases the potassium level in proportion to the clinical effect.

ACKNOWLEDGEMENTS

This work forms a part of the program, which is aided by Reumaliitto (Society for Campaign against Rheumatic Diseases) and Sigrid Juselius' Stiftelse. We are indebted to Chief of the Rheumatism Foundation Hospital, Dr. Veikko Laine, for the material, and to Miss Valma Peltola and Mr. Veikko Leppänen, M. A., Kivelä Hospital, for flame photometry. We are also indebted to Dr. Lauri Autio, Kivelä Hospital, for valuable suggestions and advice in the colloid osmotic pressure determinations.

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THE EFFECT OF INTRINSIC FACTOR IN PERNICIOUS TAPE-WORM ANEMIA

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ıe clinical picture of Addisonian perni- anemia and pernicious tape-worm ane- differs in that in the latter free hydro- ic acid is sometimes found in the gastric , whereas in the former there is always k of hydrochloric acid. As early as in , Schauman & Levander observed that ochloric acid was present more often in nts recovered or recovering from tape- n anemia than in those still suffering the disease. After recovery, the gastric in seven of their 29 patients with tape- n anemia again contained hydrochloric Later, Helander reported similar re- . He noted also decreased secretion of in during the anemic stage. Hernberg's 47) follow-up studies of patients with -worm anemia revealed that small quanti- of free hydrochloric acid were found in ost half of the patients after recovery. patient's age played no role, but the time sed after recovery was of significance. longer time elapsed, the higher were the es. Similar observations were made with rd to secretion of chymase and pepsin. ording to Hernberg, reduced quantities astric juice and its enzymes would thus r in pernicious tape-worm anemia. Siu- 's recent investigations of hydrochloric secretion revealed similar conditions; eover, he observed that the changes in the mucous membranes of the stomach showed signs of normalization during the period of recovery. The presence of hydrochloric acid in the gastric juice of some patients with tape-worm anemia suggests that it might cointain intrinsic factor as well. This is not the case in Addisonian pernicious anemia. Clinical examinations made by Hernberg (1936, 1941) show that the gastric juice of patients with tape-worm anemia may contain intrinsic factor. In some patients with tape-worm anemia — but not in all — vitamin B_{12} given by mouth (v. Bonsdorff & Gordin) may cause a relapse. This indicates a lack or relative lack of intrinsic factor in some cases of tape-worm anemia. Studies of uropepsin secretion (Lumme, Mustakallio, Telkkä, Tötterman, 1954) have shown that it persists in half of the patients with tape-worm anemia; in half the secretion is reduced like in Addisonian pernicious anemia in which normal values are never noted. The above observations favor the idea that disturbances of the secretory function of the stomach are frequent in pernicious tape-worm anemia, and secretion of intrinsic factor may simultaneously be reduced. Tests on the effect of intrinsic factor alone, without the addition of vitamin B_{12}, on the blood picture in pernicious tape-worm anemia were therefore made.

MATERIAL

The intrinsic factor used was prepared of fresh mucosa from pig stomach (kindly supplied by Biofac A/S, Copenhagen). It has been previously noted (Bastrup-Madsen & Paulsen, 1955) that this preparation of intrinsic factor in daily doses of 10—30 centigrams together with 10—30 micrograms of vitamin B_{12} raises the blood count in Addisonian pernicious anemia. As hospital diet contains vitamin B_{12} in any case and an improvement in the blood picture is observable from the change of diet alone, the patients' blood values were recorded prior to the administration of intrinsic factor to make sure that any later response was not due to changed diet or rest. The intrinsic factor preparation was examined microbiologically for possible vitamin B_{12} with the aid of the E. coli 113—3 strain with a great affinity for vitamin B_{12}. The intrinsic factor contained 0.00008 micrograms per gram of vitamin B_{12} which is but a fraction of what the daily diet contains, and thus of negligible importance.

Our material comprises 6 patients with pernicious tape-worm anemia whose reticulocyte counts after administration of intrinsic factor were compared with those of 9 patients with Addisonian pernicious anemia receiving the same preparation.

RESULTS

Intrinsic factor, 0.5 g given twice daily. Figure 1 shows that distinct reticulocytosis occurred in all 4 patients with tape-worm anemia given daily doses of 1 g of intrinsic factor. The reticulocytosis was also marked in 3 patients with Addisonian pernicious anemia; no reticulocytosis resulted in a fourth case, a man aged 45. A woman, aged 45, with tape-worm anemia, responded with a 20 per cent reticulocytosis after the administration of intrinsic factor. After anthelmintic treatment, a fresh reticulocytosis of 8 per cent occurred. Another tape-worm patient, a woman aged 47, had a reticulocytosis of 7.1 per cent after the administration of intrinsic factor and after the tape-worm

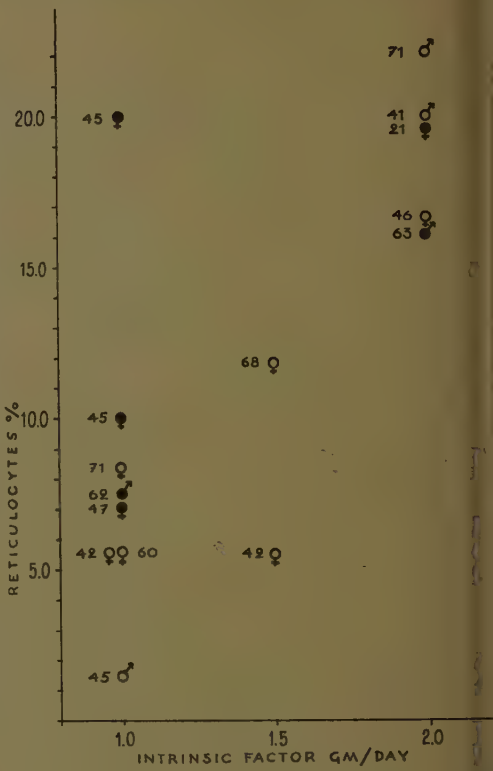

Fig. 1. The effect of intrinsic factor (with vitamin B_{12}) in pernicious tape-worm ane (♂, ♀) and Addisonian pernicious anemia (♂,

cure. Reticulocytosis, although less mar (under 5 per cent) was noted also in ot patients with tape-worm anemia in this gr after expulsion of the worm. A daily dos 1 g of intrinsic factor did not suffice for the patients with Addisional pernicious a mia. Two patients were given larger do 0.5 three times daily. Favorable respo were obtained in both cases: 11.8 per in one of the cases. This seems to indi that intensified reticulocytosis might be tained with larger doses of intrinsic fac

Intrinsic factor, 1 g given twice d Table I shows distinctly that the adminis

of 1 g of intrinsic factor twice daily
ted in higher reticulocyte counts than
er doses invoked. In a woman, aged 21,
tape-worm anemia, the reticulocytosis
ed on the seventh day after beginning
ment, and persisted over a period of 15
After anthelmintic treatment there was
slight reticulocytosis. Similar observa-
were made with regard to reticulocyto-
fter anthelmintic treatment in the case
man of 63.

characteristic common to all patients
tape-worm anemia given intrinsic factor
the fact that improved appetite and a
ng of well-being was mentioned as early
n the second or third day.

o make sure that the tape-worm patients
anemia in our series were true cases,
not patients with Addisonian pernicious
nia with tape-worm as a secondary find-
the patients treated were asked to abstain
medication on their return home. These
nts were followed-up at intervals of a
months. Their blood values improved,
h most probably would not have occur-
if there had been any question of Addi-
an pernicious anemia.

DISCUSSION

rom a study of the ages of tape-worm
ers, patients with tape-worm anemia,
patients with Addisionian pernicious
nia, Tötterman & Ahrenberg (1956)
rved that the age distribution in tape-
n anemia and Addisionian pernicious
nia was highly similar but qutie different
ng the tape-worm carriers. This suggests
tape-worm anemia may often be due to
lar internal or hereditary causes as Ad-
disonian pernicious anemia. The tape-worm might only be the trigger factor; the anemia, in consequence, arising at a younger age than otherwise.

Considering the possible endogenous causes of tape-worm anemia, it is natural to wonder whether or not a relative lack of intrinsic factor occurs in tape-worm anemia like in Addisonian pernicious anemia. In our tests with intrinsic factor alone, the effect on the blood picture in tape-worm anemia was distinctly favorable, which is a point in favor of the assumption mentioned. Simultaneously with our investigation, Kaipainen & Ohela (1956) in their studies observed that excretion of radioactive vitamin B_{12} in the urine (Schilling's) test rises when patients with tape-worm anemia are given intrinsic factor, as it does after expulsion of the worm. In Kaipainen & Ohela's tests, the increased excretion of radioactive vitamin B_{12} together with intrinsic factor was not as great as after expulsion of the worm. This may have depended on the amount of intrinsic factor given; in Schilling's test, large doses of intrinsic factor may yield higher higher values. This is to some extent substantiated by the fact that the larger doses of intrinsic factor used in our tests invoked more intense reticulocytosis.

SUMMARY

The effect of intrinsic factor on the blood picture in pernicious tape-worm anemia was measured by observation of the reticulocytosis. The results were compared with those obtained in similar tests on patients with Addisonian pernicious anemia. Distinct reticulocytosis was obtained in all cases of per-

nicious tape-worm anemia. The results seem to indicate that intrinsic factor plays a part in the origin of pernicious tape-worm anemia as well as in Addisonian pernicious anemia.

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Technique and Practical Problems

ındinav. J. Clin. & Lab. Investigation 395—397, 9, 1957.

PAPER CHROMATOGRAPHY OF FREE PORPHYRINS WITH NEUTRAL SALT SOLUTIONS

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Two methods for the separation of free rphyrins on paper have been described: idine chromatography and paper electro- oresis. The former procedure requires ee to five hours with the method of iksen (1953) and here the porphyrins ve faster the lower their number of car- xyl groups (*i. e.,* protoporphyrin first, oporphyrin last); electrophoresis requires newhat less time (With, 1956) and the rphyrins move in the opposite order (*i. e.,* oporphyrin first, protoporphyrin last). We ve found that ascending paper chromato- aphy of free porphyrins with dilute neutral t solutions gives a separation very similar that obtained on electrophoresis, and this y be achieved in less than one hour with remely simple technic. Fluorides, chlo- es, bromides, iodides and sulphates of the ali metals, calcium, and magneesium in ncentrations of about M/10 were found table, lithium chloride giving the most utiful results. The spots of the various rphyrins show, however, some tendency towards streaking, but this is counteracted by performing the procedure in an athmos- phere of ammonia vapors.

METHOD

The porphyrin solution is applied on sheets of Whatman I paper, 24 cm broad and 20 cm long, along a line 2 cm from the bottom. The solution — in ammonia, ammonia-acetone, or HCl-acetone — is brought into contact with the paper, by means of Pasteur pipettes, and the solvent is completely evaporated in a warm air current from an electric hair-dryer. The fluorescence of the spots is controllcd in Wood's light (With, 1957) and more solution applied until the spots show fluorescence of appropriate strength. The fluorescent spots should be 0.5 to 1 cm in diameter and the free space between two adjacent spots at least two cm. In addition to the spots of the test solutions, marker solutions of purified porphyrins in 2 N ammonia have to be applied to the paper for control of the run, *e. g.* uroporphyrin, coproporphyrin, and a discarboxyl porphyrin. A suitable dicarboxyl porphyrin is hematoporphyrin which is commercially available as hematoporphyrin hydrochloride from Light & Co. The marker solutions in ammonia are stable in darkneess at room temperature for several months. Their concentration is controlled by application of one drop to the paper and studying the fluorescence of the spot in Wood's light; if this is bright red, but not very intense, the concentration of the marker solution is convenient.

After the application of the spots the paper is formed as a cylinder and fixed in this form with adhesive tape at the upper end of the cylinder. The cylinder must not be closed, a free space of a

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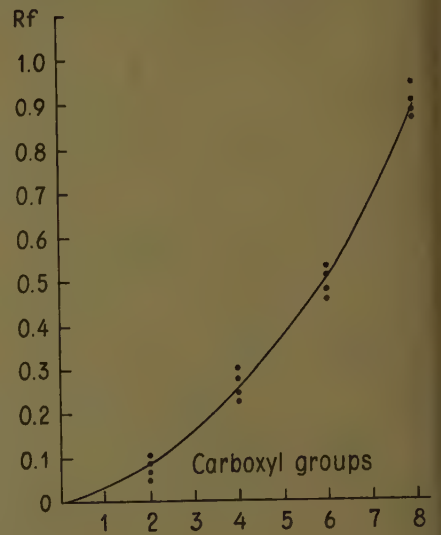
few millimeters between the adjacent edges of the paper being left. The paper cylinder is now placed around a beaker with about 10 ml conc. ammonia, in a glass dish of appropriate size (*e. g.*, a Petri dish), after which 20 ml of M/10 lithium chloride is poured into the dish. Finally, the dish with the paper cylinder is covered with an inverted glass cylinder with diameter 14—15 cm and height ca. 30 cm. Both the dish and the glass cylinder are placed on a glass plate and made airtight by silicone grease on the free edge of the glass cylinder.

The chromatograms are run at room temperature, and satisfactory separation is achieved in an hour or less. The position of the spots may be followed during the run by irradiating the glass cylinder with Wood's light.

After the run is completed the paper is removed from the tank and examined in Wood's light, and the spots are marked with a pencil as well as possible on the wet paper. Then the paper is dried on a radiator and the final drawing of the fluorescent spots carried out under Wood's light.

RESULTS

The spots are more elongated and not quite as clearly separated as those resulting from lutidine chromatography. The Rf values are for hematoporphyrin 0.05—0.09, for coproporphyrin 0.20—0.30, for 6-carboxyl porphyrin 0.45—0.55, and for urophorphyrin 0.85—0.95. If too large amounts of porphyrin are applied to the paper the spots become very elongated and tend to flow together. Spots of moderate fluorescence intensity give the best results. The relatively large variation of the Rf values makes the use of markers necessary. The method is incapable of clear separation of porphyrins with less than 4 carboxyl groups from each other. Here the lutidine method is definitely superior. The method might be employed as a descending chromatography with a run of several hours duration and this might achieve clear separation of porphyrins with 2—4



carboxyl groups, but such a separation wou be of limited interest because the lutidi method gives this more simply and rapid If the Rf values are plotted against the nu ber of carboxyl groups in a coordinate s tem and a curve is drawn, it is clearly s that there is no linear relation such as is case with the lutidine method (Nicholas Rimington, 1949). A curve from a char teristic run of a sheet with several spots each porphyrin is seen in Figure 1.

APPLICATION OF THE METHOD IN THE STUDY OF PORPHYRIA URIN

Qualitative analysis of porphyria uri may easily and rapidly be performed means of the chromatographic method scribed and the following pre-treatment the urine:

An appropriate volume of urine — 20 to 100 will as a rule be convenient — is acidified to 2—4 and adsorbed onto a 2 mm thick layer of prepared on a Büchner funnel (diameter 5—6

ilter paper covering the holes of the funnel laced in its bottom, distilled water is sucked ugh, and when the paper is sucked firmly into bottom the suction is stopped, and a suspension alc in distilled water is poured on. A little hol is dripped into the suspension to remove n, and the suction is put on again until a firm r of talc with a smoth surface covers the bottom ne funnel. The alcohol is washed away by suck- distilled water through the funnel, then the ified urine is poured on and sucked through. nay be necessary to filter the acidified urine ugh a paper filter first as it may block the filter. As a rule 80—90 per cent of the urinary phyrins are adsorbed to the talc in this way. n follows elution with HCl/acetone — 1 volume ICl and 9 volumes acetone — by suction through Büchner funnel; only a small volume of the elu- fluid has to be employed in order to get the iest possible concentration of porphyrin. The te may be directly applied to the paper and the matographic run started after appropriate por- rin markers have been applieed for control, but her concentration of the urinary porphyrin may be achieved by precipitating the HCl/acetone tion by addition of concentrated ammonia drop drop to pH about 9, collecting the precipitate filter paper, and dissolving it in as small a ume of ammonia as possible. This concentrated tion can then be applied to the paper for the matographic run.

The adsorption, eluation, and precipitation the urine is, with a little practice, easily finished within half an hour, and the whole analysis including the chromatographic run may easily be completed within two hours.

SUMMARY

A simple and rapid method of paper chromatography of free porphyrins is described based on ascending chromatography with dilute solution of neutral salts, *e. g.* M/10 lithium chloride at room temperature in an ammonia atmosphere. Separation of porphyrins with 4 or more carboxyl groups is achieved in less than one hour. After a simple and rapid pre-treatment the method may be used in the analysis of porphyria urines.

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PURE UNEQUIVOCAL UROPORPHYRIN III

SIMPLIFIED METHOD OF PREPARATION FROM TURACO FEATHERS

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It was pointed out by Waldenström, Fink & Hoerburger (1935) that urophorphyrin esters isolated from urines from patients with acute porphyria differ from uroporphyrin I ester, and they presented strong evidence indicating that these porphyrin esters, now known as Waldenström esters, are uroporphyrin III esters. This was, however, strongly doubted by Grinstein, Schwartz & Watson (1945) who were able to isolate uroporphyrin I ester from several Waldenström esters by means of chromatographic fractionation. Since then the composition of Waldenström esters has been a point of controversy, mainly because of the lack of a source of pure, unequivocal uroporphyrin III.

Rimington (1939) found that the uroporphyrin of turacin, the red pigment of the flight feathers of the *Musophagidae,* is uroporphyrin III, and this was confirmed by Nicholas & Rimington (1951). But Watson & Berg (1935) were unable to isolate homogenous and unequivocal uroporphyrin III from various samples of turacin; their communication is, however, given in a foot-note without any details. Other investigators have so far not published studies on the question, presumably because of the laborious and time-consuming technic. A simplification of the method of preparation of pure uroporphyrin from turaco feathers is therefore desirable and will presumably stimulate research.

Below, a simple technic for preparation of uroporphyrin ester from turaco feathers is described, and the ester is identified as uroporphyrin III ester in full confirmation Rimington's statements.

Fischer & Hilger (1924) believed t uroporphyrin from turacin was uroporph rin I, a fact presumably responsible for t failing interest in Rimington's import findings. Furthermore, turacin is a urop phyrin copper complex where the porphy is very firmly bound and correspondin difficult to liberate. Because Hill (192 succeeded in splitting artificial protoporp rin-Cu complexes with concentrated s phuric acid, I tried this on turaco feath and found that the porphyrin was libera to judge from the fluorescence. Riming (private communication) has since inform me that his collaborator J. Falk used same method for liberation of the tura porphyrin in 1954, but Falk's technic cluded de-fattening, treatment with H_2S at room temperature, pouring into ice-wa precipitation of the porphyrin on neutral tion, and drying of the precipitate before esterification. And all these procedures omitted in the technic described below wh begins with esterification.

An important point is that uroporphy contrary to protoporphyrin, does not un go any change on addition of concentr sulphuric acid (Fischer & Zerweck, 19

PROCEDURE

1. Extraction and esterification.

The red parts of the feathers are separated the black and colorless and weighed. A mea volume of pure, concentrated H_2SO_4, sufficie

et" them thoroughly, is added and mixing per-
med with a glass rod. After ca. one hour a
ume of absolute methanol, sufficient to pro-
ce a 5 per cent v/v sulphuric acid-methanol
h the volume of H_2SO_4 employed, is added
tiously in small portions under stirring with a
ss rod (heat development). Example: 1.425 g
thers, 20 ml H_2SO_4, 380 ml methanol. After
ving over night in a beaker covered with a watch
ss the solution is decanted into a separatory fun-
, neutralized (congo paper) with saturated so-
m acetate, after which extraction with chloro-
m (pharmacopoiea quality) follows; addition of
tilled water may be required to bring about phase
aration. The completeness of the extraction is
trolled with fluorescence in Wood's light, most
venient with a portable lamp (With, 1957). The
led $CHCl_3$ extracts are washed with distilled
ter twice, with 2 N ammonia once, and again
h water twice. All phase separations in extrac-
ns and washings are remarkably clear and rapid.
e washed $CHCl_3$ extract is dried by filtering
ough a double layer of paper filter wetted with
shed, dry $CHCl_3$ and addition of a few granules
anhydrous calcium chloride.

2. *Chromatographic purification.*

After evaporation at room temperature the ex-
ct is dissolved into as small a volume of $CHCl_3$
armacopoiea quality) as possible and poured on
short column of magnesium oxide (height ca. 2
, width 1.5—2 cm) in a chromatography tube.
e MgO employed has to be of grade III accord-
to Brockmann & Schodder (1941); the BDH
eparation "for chromatography" being suitable.
e column is developed with $CHCl_3$ (pharma-
oiea quality) and is also prepared from MgO
spended in this solvent — which is employed
cause of its content of a little alcohol. In the
rse of the development the porphyrin passes
o the filtrate while a dark, non-fluorescent zone,
-2 mm broad, remains at the top of the column.
me porphyrin is retained on the lower part of
e column as shown by a diffuse pink fluorescence.
After this purification chromatography the ex-
ct is again evaporated and chromatography on
similar column of MgO, this time prepared from
suspension in freshly washed and dried $CHCl_3$,
performed. Height of column 3—5 cm, width
—2 cm, development first with freshly washed,
y $CHCl_3$, then with the same solvent containing
—0.2 per cent v/v of absolute methanol. It is
nvenient to cover the top of the column with a
er of acid-washed sea sand. On development with
HCl_3 two zones appear, an upper brownish smal-
one and a broader red one ½—1 cm below —
lled B and A respectively. During development
th the $CHCl_3$-methanol A passes quantitatively
o the filtrate while B remains on the column.
may be partially eluated with 5 per cent methanol
in $CHCl_3$, but for its quantitative liberation sul-
phuric acid-methanol is necessary.

Further details concerning chromatography tech-
nic are found by Nicholas (1951).

The B zone was studied by Nicholas & Riming-
ton (1951) and consists of an octacarboxylic por-
phyrin different from uroporphyrin. The uropor-
phyrin is found in the A zone.

3. *Crystallization.*

The A fraction is dissolved in as little $CHCl_3$
as possible and a surplus of absolute methanol added
in a beaker at room temperature. In about ½ hour
crystallization takes place, delicate floccules ap-
pearing slowly. This is in sharp contrast to the
behavior of uroporphyrin I from bovine porphyria
urine under similar circumstances; this porphyrin
ester crystallizes practically immediately and in
profuse amounts. The crystals are centrifuged down
and washed several times with absolute methanol.
Re-crystallization was performed in the same way
or from $CHCl_3$/petrol ether which gives a more
rapid crystallization.

IDENTIFICATION OF TURACO A PORPHYRIN AS UROPORPHYRIN III

By means of paper chromatography (Erik-
sen, 1953) the crystals — after hydrolysis
for 36 hours with 7 N HCl — were shown
to consist of octacarboxyl porphyrin and in
some cases a faint trace of heptacarboxyl
porphyrin.

The melting point of the crystals was de-
termined in a Gallenkamp micro-melting
point aggregate. The primary crystals melted
at 250—260°, and on re-crystallization the
M. P. increased to 260—265°.

For spectrophotometric studies 5—10 mg
crystals were transferred to a 10 ml measur-
ing flask and 2 ml concentrated H_2SO_4
added; after addition of a little water com-
plete dissolution took place due to the heat
developed. Cautious dilution with distilled
water followed and, after cooling, adjustment
of the volume and appropriate mixing. Then
spectrophotometric measurement was carried
out after dilution 500—1,000 times with 0.5
N HCl. The Soret maximum was 405 mμ
with an $E\,\frac{1\%}{1\ cm}$ of about 6 500 for the pure
re-crystallized preparations. Readings were
performed in a Hilger Uvispec instrument
with quartz prism.

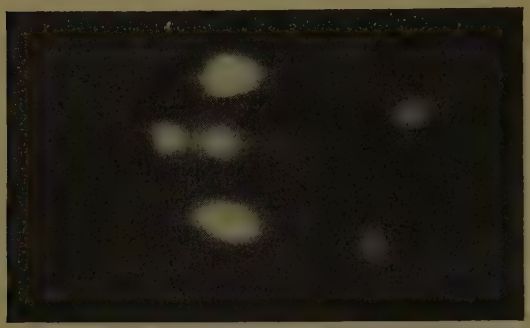

Fig. 1. Lutidine chromatography of decarboxylized specimen. By courtesy of C. Rimington (see text).

Thus our findings point to a clear difference from uroporphyrin I ester which shows a different behavior on crystallization (cf. above) and melting point of 290—295° for pure preparations — often confirmed in our laboratory with preparations from bovine porphyria urine. Our findings are in agreement with the figures of Rimington.

Conclusive demonstration of the identity of turaco A ester with uroporphyrin III ester was obtained on decarboxylation according to Fischer & Zerweck (1924), one sample being subjected to this treatment in Rimington's laboratory, another in the Copenhagen University Chemical Laboratory through the courtesy of Professor K. A. Jensen. From both samples coproporphyrin III ester was isolated in crystalline condition.

In Rimington's laboratory 370 μg of the ester was heated to 180—183° in 1 per cent HCl for 3 hours in sealed tubes. The coproporphyrin was transferred to ether, evaporated and esterified. The yield was 141.8 μg, corresponding to 51 per cent of the amount expected theoretically. A sample was subjected to special lutidine chromatography giving separation of copro I and III isomers and showed a very strong spot of copro III, a fairly strong spot in the tricarboxylic porphyrin position, but *absolutely no copro I.* A photograph of the lutidine strip in Wood's light is seen in *Figure 1.* Here A and C are the Turaco porphyrin spots, B the coproporphyrin markers: a coproporphyrin I, b coproporphyrin III, and c coproporphyrin II; d is the tricarboxylic porphyrin spots.

The remainder of the turaco A ester was crystallized from hot chloroform-methanol and formed small rosettes and prisms melting at 146—148° solidifying on cooling and re-melting at 172—174° This solidifying and re-melting at a higher temperature is very characteristic of coproporphyrin III

The decarboxylation performed in Copenhagen gave less material and lower purity. The coproporphyrin was extracted from the HCl-solution with ether-acetic acid after neutralization. No fluorescence remained in the aqueous phase. The porphyrin was completely extracted from the ether with 0.1 N HCl and adsorbed on talc. The dried adsorbate was esterified with 5 per cent v/v H_2SO_4 methanol and extracted with chloroform. Crystallization from hot $CHCl_3$/methanol followed. Only a few crystals appeared after long standing and they were mixed with greasy material. But the consisted of rosettes of short, prismatic needles radiating from a center, the form characteristic of coproporphyrin III. Melting point determination showed mollification at 120° and complete liquefaction at 140°. Solidifying on cooling did not take place due to the greasy contamination.

The decarboxylations show beyond question that turaco A porphyrin is uroporphyrin III. A further confirmation of this was obtained in Rimington's laboratory with paper chromatography of the turaco A ester according to Falk & Benson's (1953) dioxane method. With this method the sample behaved like uroporphyrin III although with some "tailing".

QUANTITATIVE PORPHYRIN ANALYSIS ON THE FEATHERS

The H_2SO_4-methanol extract of the feathers was diluted 1/100 with 0.5 N HCl and subjected to spectrophotometric reading (With, 1955). The total porphyrin was calculated as uroporphyrin and found to be between 3 and 5 per cent of the weight of the red parts of the feathers in all the four batches of feathers studied. Thus the porphyrin content of this porphyrin source is at least as high as that of purified hemoglobin!

STUDIES ON EXTRACTION WITH ETHYL ACETATE

According to Waldenström *et al.* (1953) uroporphyrin III is soluble in ethyl acetate at pH ca. 3.2 while uroporphyrin I is not. Re-investigation by Dresel & Tooth (1955) in Rimington's laboratory showed, however

t both uroporphyrins were quantitatively racted into ethyl acetate from aqueous ıtions at pH 3.0—3.5 provided these were ɔngly buffered — a fact rarely mentioned the literature although the findings of ldenström *et al.* are quoted extensively. ıdies in our laboratory with uroporphy- s I (from bovine porphyria urine) and (from turacin) fully confirmed the re- ts of Dresel & Tooth (obtained with a ldenström porphyrin). The necessity of rking with strongly buffered solutions — mentioned by Waldenström *et al.* — was ious because the porphyrins flocculated m solutions in dilute HCl when brought ɔH 3.0—3.5 as described by Waldenström *l.*; uroporphyrin III flocculated somewhat er than uroporphyrin I. This and the t that the uroporphyrin I preparation om the patient Petry) of Waldenström *l.* most likely was considerably purer than ir preparation of uroporphyrin III (a ldenström porphyrin) may be the expla- ion of their findings. In our laboratory lition of uroporphyrin I and III to normal ne followed by extraction with ethyl ace- e at pH 3.2 (secured by buffer) showed t both were extracted identically.

SUMMARY

A simplified method for preparation of ɔporphyrin from turaco feathers is pre- ted. The turaco uroporphyrin is shown be unequivocal uroporphyrin III differing arly from uroporphyrin I in the crystal- tion from $CHCl_3$/methanol and the melt- point of its ester and in giving copro- phyrin III on decarboxylation. Extraction dies with ethyl acetate revealed no differ- es between uroporphyrins I and III.

ACKNOWLEDGEMENTS

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COMPARISON BETWEEN TOTAL CO_2-CONTENT ("TOTA... CO_2") IN PLASMA/SERUM FROM BLOOD COLLECTED WITH OR WITHOUT PARAFFINE OIL

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Paraffine oil, usually employed as a protective measure against loss of carbon dioxide from blood, is not ideal; partly because carbon dioxide is not insoluble in paraffine oil (on the contrary it is more soluble in paraffine oil than in water, according to Kubie (1927), and partly because it is troublesome to rinse the oil from the tubes and pipettes. In addition it is often difficult to pipette off the plasma (serum) without getting paraffine oil in the pipette.

The problem of dispensing with paraffine oil, when sampling blood for analysis of carbon dioxide, has not received much attention in the literature. Peters & Van Slyke (1932) only mention that one has to use the anaerobic technique and simultaneously point out that if the blood sample is kept under paraffine oil, no greater loss of carbon dioxide takes place, while centrifuging involves a considerable loss, even when protected by paraffine oil.

Harrison (1947) on the other hand, does not use paraffine oil, arguing that stoppering the tubes gives sufficient protection.

For that reason it is of interest to ascertain experimentally if one can dispense with paraffine oil and rely on stoppering the tubes as a sufficient measure for prohibiting the escape of carbon dioxide from the blood.

METHODS

All investigations have been carried out by the author personally. Total-CO_2 content in plasma/serum was determined according to Van Slyke and Neill's (1924) manometric technique. The two evacuation chambers employed and the blood pipettes were weighed out with distilled water. The volu... of the chamber at the 2.00 ml mark was determin... to 1.992 ± 0.012 ml.

1) *Sampling of blood:* A short sharp-eg... needle No. 12 (inner diameter: 1.2 mm) 3.5 ... long, was used, mounted on a 20 ml Record syring... filled with 2 ml sterile paraffine oil. The sta... on the arm was loosened immediately after t... vein was punctured, the syringe was filled wi... a slight pull in the barrel, after which a ne... syringe without paraffine oil was filled. A 5 ... long needle was immediately mounted on ea... syringe, 5—6 drops of blood were discarded a... the blood in the first syringe was passed dow... under a 2 cm layer of paraffine oil in a centr... fuging tube (10 × 2.5 cm, volume 25 ml). T... blood in the other syringe was passed down... a similar centrifuging tube without paraffine o... After carefully mixing both tubes were stoppere... with a rubber stopper. All the tubes contained ... mg sodium fluoride and 1.25 mg heparin in ea... tube. 0.5 ml of the following solution was add... to the tubes and thereafter dried at 37° C: 3 gr... sodium fluoride was dissolved in 80 ml distill... water, 5 ml 5 per cent Heparin Leo added a... diluted to 100 ml by distilled water.

About 10 minutes later the tubes were centr... fuged for 5 minutes in a centrifuge with a free... swinging head with a speed of 2000 r. p. m. Imm... diately afterwards the analyses were started.

2) *Determination of the reabsorption fact... ("i-factor"):* The determination was made accor... ing to Van Slyke & Sendroy (1927) by analysis ... three solutions containing 20.00, 12.50 and 6.... mEq/l Na_2CO_3 p. a. (Merck) (desiccated *in vac...* for three days) and glass distilled water, free ... CO_2, corresponding to 40.00—25.00 and 12.... mEq/l CO_2.

The factor for the different solutions was 1.0... 1.014 and 1.017 respectively. The mean value w... 1.015.

3) *Accuracy of the method:* As a control of t... method four different solutions of Na_2CO_3 we... analysed on four consecutive days. The differen... in per cent between the theoretical value and t... value found was for all four solutions less th... 0.6 per cent (Table I).

Table I. *Results of total-CO_2 determinations on known solutions of sodium carbonate*

ate	Total-CO_2 mEq/L computed	Total-CO_2 mEq/L found	Diff.
3	40.00	39.95	+0.05
3	»	40.23	—0.23
3	»	40.18	—0.18
3	»	40.09	—0.09
11	25.00	24.91	+0.09
11	»	24.98	+0.02
11	»	24.89	+0.11
11	»	24.92	+0.08
9	20.00	19.99	+0.01
9	»	20.04	—0.04
2	12.50	12.49	+0.01
2	»	12.52	—0.02
2	»	12.55	—0.05
2	»	12.57	—0.07

In 10 single analyses of the same solution of ium carbonate (25.00 mEq/l CO_2) the standard riation was found to be ±0.13 mEq/l and stan-rd error ± 0.043 mEq/l. In another series of periments the accuracy of the method was elu-ated by double determinations on 15 normal and pathological plasmas. The standard deviation D.) for the normal plasmas was ± 0.13 and 0.12 for the pathological plasmas (total-CO_2 rying from 6.20 to 36.8 mEq/l CO_2). The corresponding standard error (e) for the series was ± 0.09 and ± 0.08 respectively.

RESULTS

Comparison between total-CO_2 in plasma and serum:

No significant difference between total-O_2 in plasma or serum could be shown, as pears from Table II.

The calculated t-value (1.57) is signifi-ntly lower than the computed t-value for ne degrees of freedom at the 1 per cent vel (3.25).

Table II. *Results of determinations of total-CO_2 in plasma and serum collected anaerobically from 10 persons.*

No.	Total-CO_2 mEq/L Heparin plasma	Total-CO_2 mEq/L Serum	Diff.
1	31.35	30.92	—0.43
2	27.54	27.48	—0.06
3	27.39	27.14	—0.25
4	26.97	27.21	+0.24
5	26.90	27.26	+0.36
6	26.90	26.96	+0.06
7	26.84	26.82	—0.02
8	26.30	26.53	+0.23
9	25.29	24.96	—0.33
10	25.28	25.37	+0.09

Standard error e = ±0.07
t-value = 1.57

Comparison between total-CO_2 in plasma collected with and without paraffine oil in stoppered tubes:

A total of 25 samples, partly from normal rsons, partly from patients, were analysed th double determinations with and without raffine oil in stoppered tubes. A sample with paraffine oil was first analysed, next a sample without paraffine oil and so on. The results appear in Fig. 1.

The values are repreesented by a straight line passing through origo with an angle of 45°. The greatest differences was 0.37 mEq/1. Thus one obtains just as exact results with the blood collected in stoppered tubes without paraffine oil as if collected under paraffine oil.

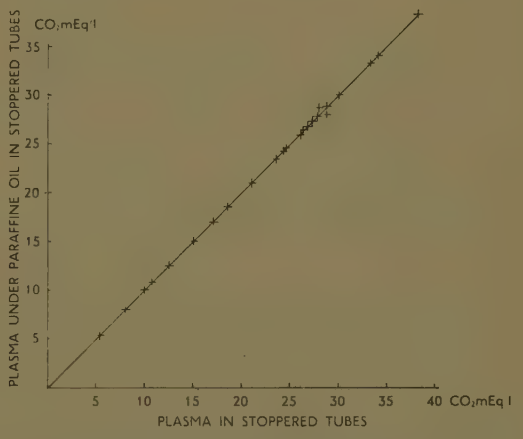

Fig. 1. Comparison between total-CO_2 in plasma collected with and without paraffine oil in stoppered tubes.

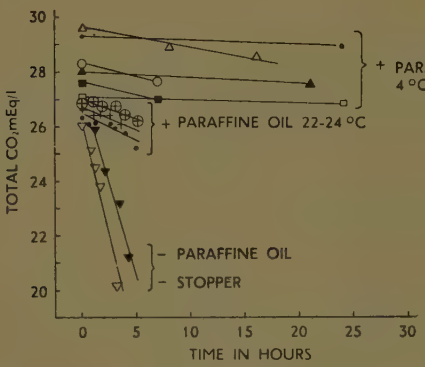

Fig. 2. Comparison between total-CO_2 in heparin plasma stored at 4° C and at 22—24° C anaerobically.

Loss of CO_2 from plasma in stoppered and non-stoppered tubes stored with as well as without paraffine oil:

The effect of the storage time on the loss of CO_2 from the samples was investigated when stored at 4° C and at 23° C. The samples, which had been stored in the refrigerator, were allowed to stand ¾ of an hour for re-warming to room temperature. The results appear in Fig. 2.

As seen, the loss of CO_2 from samples which had been stored in the refrigerator under paraffine oil in stoppered tubes, was about 0.2—0.5 mEq/l in 5 hours, the greatest loss within 24 hours being 1 mEq/l.

The loss of CO_2 from samples stored at room temperature was about 1 mEq/L in 5 hours. On the other hand, the loss of CO_2 from samples stored at room temperature without paraffine oil in tubes without stopper was great, about 6 mEq/L in 2½—4 hours.

The effect of speed and duration of centrifugation on the total-CO_2 content of plasma collected under paraffine oil:

From Fig. 3 it appears that the loss of CO_2 from the blood samples after centrifugation for 10 minutes was 0.9—1.0 mEq/L independent of speed of centrifugation.

The determinations on uncentrifuged samples were made after spontaneous sedimentation of the corpuscles for 5—10 minutes. Between each determination the corpuscles and plasma were carefully mixed. Five minutes' centrifugation at a speed of 2000 r. p. m. only caused a loss of CO_2 of about 0.3 mEq/L.

DISCUSSION

In the determination of the reabsorption factor ("i-factor") on known solutions of sodium carbonate, this factor showed some variation (from 1.014 to 1.017). The factor is a measure of the reabsorption of CO_2 taking place in the reaction mixture, when the gas-liquid volume in the evacuation chamber is changed from 50 ml to 5.5 ml. this factor was originally determined by Van Slyke & Neill (1924) to 1.014 and later by Van Slyke & Sendroy (1927) to 1.01. Factors for calculating total-CO_2 content of plasma/serum from the partial tension of CO_2 (in mm Hg) read on the manometer of the apparatus, the temperature and the factor 1.014 have been published by Van Slyke & Neill (1924) and for the factor 1.017 by Peters & Van Slyke (1932). The author

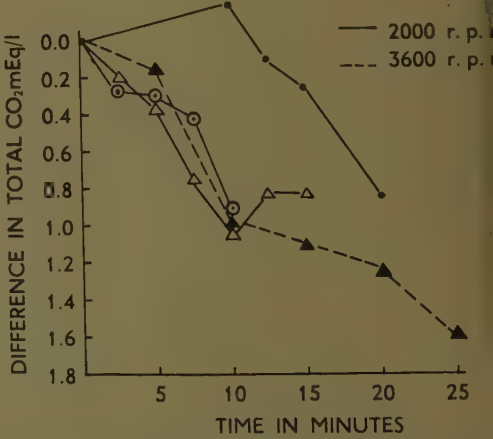

Fig. 3. The influence of the time of centrifugat on the content of CO_2 in heparin plasma from bl collected anaerobically.

or 1.015 is between these values, the difence being negligible, and has not been en into consideration. The value 1.017 has stantly been used for the calculation of total CO_2. With respect to the accuracy he method employed, Van Slyke & Neill 924) determined the standard deviation to 0.09 mEq/L on double determinations; s accuracy corresponds to the accuracy nd in this work, which was practically ependent of the total-CO_2 content.

No difference in total-CO_2 content between sma and serum could be expected; this s experimentally confirmed.

The use of oxalate as an anticoagulant is ssibly a source of error, partly because tain oxalate preparations contain small ounts of carbonate and partly because nges can arise in the water-electrolyte tribution between plasma and cells (Eisen-n, 1927). This is valid even for the use citrate. For the purpose in question hepa- is considered to be the best anticoagulant, ng neutral and having no osmotic effect. The addition of fluoride to the tubes re-ains glycolysis and the growth of bacteria the blood samples; on storage a "respira-n" takes place in the erythrocytes with nsumption of oxygen and formation of bon dioxide, for which reason plasma rum) has to be pipetted off within one ur. The cooling of the samples further in-its the glycolysis and the loss of carbon xide, which appears from Fig. 2.

According to Fig. 1 it was not possible to nonstrate any difference in the total-CO_2 plasma from blood collected with or with- paraffine oil if the tubes were stoppered, which reason the strictly anaerobic tech-ue, earlier commonly used, can be aban-ned, if the tubes are entirely filled and mediately stoppered after the sampling. The results agree with Harrison's recommendations (1947) even though he does not refer to any experimental material.

SUMMARY

1) No difference between total-CO_2 content in plasma from blood collected with or without paraffine oil in stoppered tubes could be demonstrated by investigation of 25 samples with normal, low and high total-CO_2 content.

2) There is no difference between the concentration of total-CO_2 in plasma and serum, both collected under paraffine oil.

3) A certain loss of carbon dioxide takes place from blood samples stored under paraffine oil, depending on the time and temperature of storing. At 4° C the loss amounts to 0.3—0.5 mEq/L in 5 hours and at 23° C to about 1 mEq/L.

4) On centrifugation of the blood samples under paraffine oil in stoppered tubes, a loss of CO_2 takes place, depending on the time of centrifugation; when centrifuging for 5 minutes at room temperature (2000 r. p. m.) this loss amounts to 0.3 mEq/L.

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Letters to the Editors

Scandinav. J. Clin. & Lab. Investigation 406, 9, 1957.

ALKALINE PYRIDIN REACTION IN SERUM IN TRICHLORETHYLENE POISONING

A small part of trichlorethylene occurring in the blood during trichlorethylene poisoning is oxidized to trichloracetic acid and slowly excreted in the urine. This acid may be demonstrated in urine by the alkaline pyridin reaction, which is based on Fujiwaras colour-reaction of aliphatic chlorinated hydrocarbons. The reaction is modified for examination of urine by Forssman (1945) and Ahlmark & Forssman (1951).

As far as may be seen from the literature, trichloracetic acid has not been demonstrated in serum. The alkaline pyridin reaction may be modified for examination of serum in the following way:

PROCEDURE

The serum is examined directly or after precipitating the proteins of serum by mixing 1 part of serum, 8 parts of n/12 sulfuric acid and 1 part of 10 per cent sodium wolframate, and filtration after standing for 10 minutes (Hawk, Oser & Summerson, 1950). 1 ml serum or 1 ml filtrate, 0.4 ml pyridinum purum and 0.6 ml 50 per cent NaOH are mixed thoroughly. A few minutes later the mixture is heated in a water bath to about 80° C for 5 minutes and afterwards cooled down for 2 minutes. When trichloracetic acid is present, the pyridin zone gets red to red-orange on account of the admixture of the serum colour, red-lilac when examining filtrate of serum.

CASE REPORT

March 22, 1957, a 59 years old man by mistake drank about 15 ml of trichlorethylene, immediately followed by a burning feeling in throat and esophagus. In about half an hour he got drowsy, and 1—2 hours later miosis, nystagmus and hyporeflexia appeared. The drowsiness increased, palpitations and dizziness occurred. Next day these clinical symptoms and signs had disappeared, but the electrocardiogram showed low T waves in t conventional extremity leads. The electrocardi gram was normal before and one week after toxication.

About 5 hours after ingestion the first samp of urine and serum were examined. The alkali pyridin reaction was positive in both. Regul examinations showed positive reaction in serum a urine for 27 days. The concentration in urine w as follows: April 5, 1957: about 4 mg/100 m April 9, 1957: about 1 mg/100 ml. (Performed Helseavdelingen, Eidanger Salpeterfabrikker, He øya, Norway.)

DISCUSSION

The demonstration of trichloracetic ac in serum proves that trichlorethylene is ox dized in the blood. The occurrence of trichlo acetic acid in serum for 27 days indicat that trichlorethylene is retained in the org nism, and a small part of the trichlorethyle is by degrees oxidized and excreted in t urine.

Most trichlorethylene poisonings are chr nic and caused by inhalation. It is not possib to demonstrate trichloracetic acid in the uri until 3—4 hours after exposure (Ha marsten, 1955). When a patient with acute trichlorethylene intoxication is broug to hospital, the type of intoxication may determined somewhat earlier by analysis serum.

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SEPARATION AND QUANTITATION OF PORPHYRINS EMPLOYING AN EDTA BUFFER SYSTEM

Several reports have recently appeared cribing the application of paper electro-resis to the study of porphyrin pigments eikel, 1955; Larsen, Melcer & Orten, 5; With, 1956). The value of this tech-ue as a method for the separation of free phyrins with four or more carboxyl ups has been pointed out. However, the lication of paper electrophoresis to the ntitative estimation of individual porphy-s has resulted in recoveries ranging from ow 50 per cent (With, 1956) to 90 per t (Heikel, 1955).

This report describes the use of an ethy-e diamine tetra-acetic acid (EDTA) buf-The use of this buffer in the electro-retic separation and quantitation of por-rins has consistently resulted in signi-ntly higher porphyrin recoveries than n any other buffer system we have in-igated.

METHOD

lectrophoresis of the porphyrins was carried on Whatman No. 3 mm filter paper strips, m × 3 cm. A Durrum-type cell (capacity eight s) was employed throughout this investigation. t strips were used at each run. The EDTA er was prepared by making a 0.05 M solution he disodium salt of ethylene diamine tetra-c acid dihydrate, analytical reagent grade. The was adjusted to 8.6 with 10N NaOH. Approxi-ly 0.5 ml of 10N NaOH was required per ml of 0.05M EDTA. The strips were dipped the EDTA buffer and the excess buffer re-ed by clotting between two large filter paper . The wet strips were placed in the cell and ibrated for 1 hour at 5° ± 1° C at 200 volts. he end of an hour the porphyrin solutions, in ammonium pyridine (13 parts of 3 per cent ammonium hydroxide to 1 part of pyridine) (Chen & Bergren, 1957) were applied in volumes ranging from 0.03 ml to 0.1 ml. Aliquots larger than 0.1 ml resulted in wide trailing bands. After application of the samples, electrophoresis was carried out for 4 hours at 200 volts at 5° ± 1° C. The porphyrins were then located under ultra-violet light and the individual red fluorescent bands cut out of the wet paper and placed in a test tube containing 6 ml of HCl. The concentrations of the HCl employed for elution were 0.15N, 0.55N and 0.5N, respectively, for copro-, proto-, and uroporphyrin. The tubes were shaken intermittently for 20 minutes, then centrifuged to pack the pulp and fibers. An aliquot of the supernatant was taken for spectrophotometric analysis on a Beckman spectrophotometer. The corrected calculation constants of With (1955) were employed in determining the porphyrin concentrations.

RESULTS AND DISCUSSION

Over a period of time we have investigated numerous buffering systems for the separation of porphyrins by paper electrophoresis. Porphyrin separation could be accomplished by most of these buffering systems at a pH of 8.2 to 9.1. However, unpredictable recoveries of porphyrins, varying from 30—90 per cent, were obtained. Employing the EDTA buffer as outlined above, recoveries for uro-, copro-, and protoporphyrin were 96 ± 3 per cent, 87 ± 1 per cent and 87 ± 2.5 per cent, respectively. The mobilities were 6—6.5 cm for uroporphyrin, 0—0.2 cm for coproporphyrin and no movement for protoporphyrin. The amount of porphyrin applied to the paper strips varied from 4.5 to 0.25 μg for uroporphyrin, 2.7—0.27 μg for coproporphyrin and 2.3—0.9 μg for

protoporphyrin. The amount of porphyrin applied to the paper did not affect the percent recovery of that porphyrin.

The mobility of the porphyrins in EDTA buffer was the same as with barbital buffer under identical conditions. Mobility was increased by substituting for EDTA buffer alone a 1:4 EDTA-barbital mixture (one part 0.05M EDTA buffer pH 8.6: four parts 0.05M barbital buffer, pH 8.6). With this mixed buffer, uroporphyrin moved 10 cm, coproporphyrin moved 1 cm and protoporphyrin remained at the origin. Recoveries were the same as with EDTA alone.

Both purified porphyrin extracts and hydrolyzed porphyrin esters were employed in this investigation. Uroporphyrin extracts were obtained from four cases of acute porphyria. Coproporphyrin extracts were obtained from cases of acute porphyria and from cultures of Rh. spheroides. Protoporphyrin was obtained commercially. Hydrolyzed esters of copro-III and uro-I were employed in recovery experiments and as control markers. No difference was found in the mobility or percentage recovery of hydrolyzed methyl esters and purified extracts by this technique.

The action of EDTA in increasing the porphyrin recovery after electrophoresis may not be related to its buffering qualities b to some other effect, possibly its metal ch lating action. This problem is now und investigation.

SUMMARY

The use of an EDTA buffer system the paper electrophoretic separation of po phyrin pigments is described. The recove of uro-, copro-, and protoporphyrin, det mined spectrophotometrically, was 96 ± per cent, 87 ± 1 per cent, and 87 ± 2.5 p cent, respectively.

ACKNOWLEDGEMENT

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ıdinav. J. Clin. & Lab. Investigation 409—410, 9, 1957.

RELIABILITY OF BARKER'S METHOD FOR PBI IN SERUM

Ve were interested in the following stions: (1) whether the calculation me- l by Barker (1951) can be theoretically epted and (2) whether the addition of the ternal standard" could be safely aban- ed. Barker himself points out that the ılts do not agree with those obtained lying standard curves.

Barker's method is based on the assump- that the concentration of iodine [I] is ctly proportional to the velocity (v) of reaction $Ce^{IV} \rightarrow Ce^{III}$, which should be of first order. Thus (subindices t, o indicate time)

$$[I] = c \cdot \frac{1}{t} \cdot \ln \frac{[Ce^{IV}_o]}{[Ce^{IV}_t]}$$

en the [CeIV] is expressed as per cents ransmission (T), and a new constant k pplied

$$[I] = \frac{k}{t} \cdot \log \frac{2 - \log T_o}{2 - \log T_t}$$

numerical values for k were deter- ed in Barker's conditions as follows:

I	k
0.02 μg	147
.04	160
.06	165
.08	168
.10	170

dependence on the concentration is to ıoted.

Table I. *Effect of Barker's calculation.*

Assumed in the analysis		Calculated according to Barker
conc. μg/100 ml	amount μg	conc. μg/100 ml
1	0.004	1.05
2	0.008	1.96
3	0.012	2.85
4	0.017	3.85
5	0.021	4.96
6	0.025	5.90
7	0.030	7.05
8	0.034	8.17
9	0.038	9.20
10	0.042	10.3
11	0.047	11.4
12	0.051	12.6
13	0.055	13.8
14	0.060	15.0
15	0.064	16.7

$k = 160$, $T_o = 24.0$.

Table I was compiled using $k = 160$ and $T_o = 24.0$. T_t was calculated according to the formula (2) for assumed concentrations of iodine and then T_t' was calculated similarly for the respective sample where the "internal standard" of 0.04 μg had been added. Barker's second assumption is that the linear increase in the transmission of CeIV-solution is proportional to the amount of iodine and the "Barker-value" $[I_B]$ is calculated accordingly (formula 3).

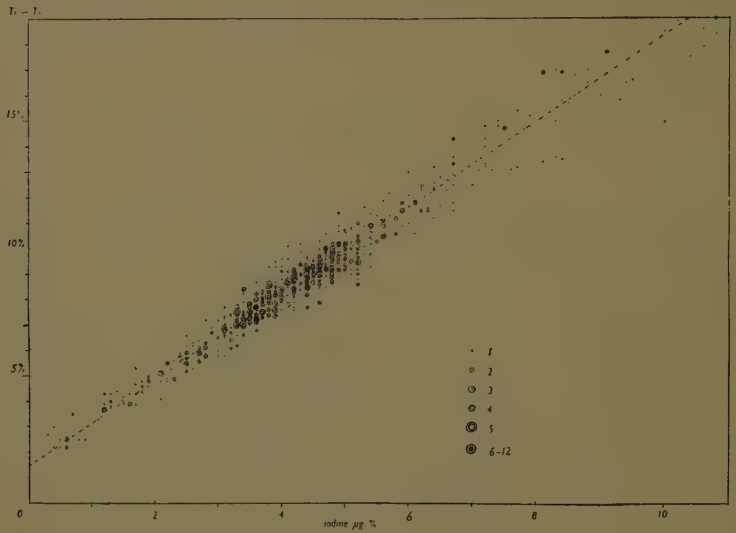

Fig. 1. $T_t—T_o$ plotted agai the iodine concentration (Barker's method). The nu ber of confluent points is dicated. The line of regress is fitted visually (see the tex The risk of 1 µg/100 ml viation is 1 per cent. (The points > 10 µg/100 ml omitted from the graph.)

Table II. *As in Table I, but the values of k estimated from the data.*

Assumed in the analysis		k used		Calculated according to Barker
conc. µg/100 ml	amount µg	for T_t	for T'_t	conc. µg/100 ml
3	0.012	140	163	3.46
6	0.025	151	166	6.52
9	0.038	159	167	9.81
12	0.051	163	169	12.7
15	0.064	166	170	17.7

T_o = 24.0; for symbols T_t and T'_t see the text.

$$(3) \qquad [I_B] = \frac{T_t—T_o}{T_t'—T_t} \cdot 0.04 \ \mu g$$

In Table II the variation of the value of k is accounted for in some examples (the values of k are estimated from the data present above).

To show the effect of occasional errors routine conditions the values of $T_t—T_o$ we plotted against the respective iodine conce trations (by Barker's method). An arbitra plot of successive determinations is present in Fig. 1. The risk of 1 µg/100 ml error omission of the internal standard is in t plot 1 per cent, providing that the inter standard would eliminate all occasio errors. The addition of iodine is necessa to check each series generally.

In conclusion, (1) Barker's method calculation causes about + 0.5 µg/100 error at usual PBI concentrations and the risk in omission of the internal standa from individual samples is small in care routine.

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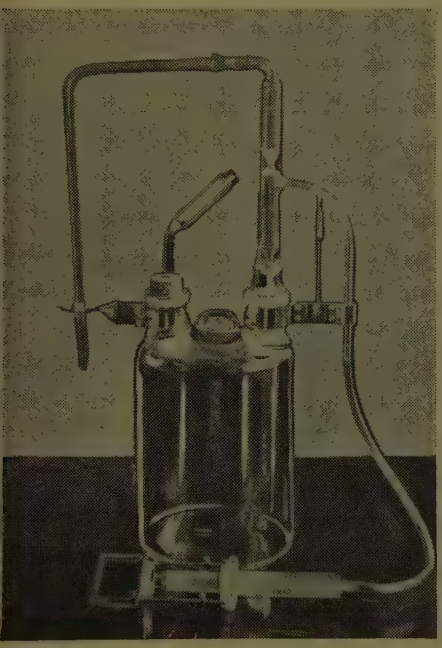

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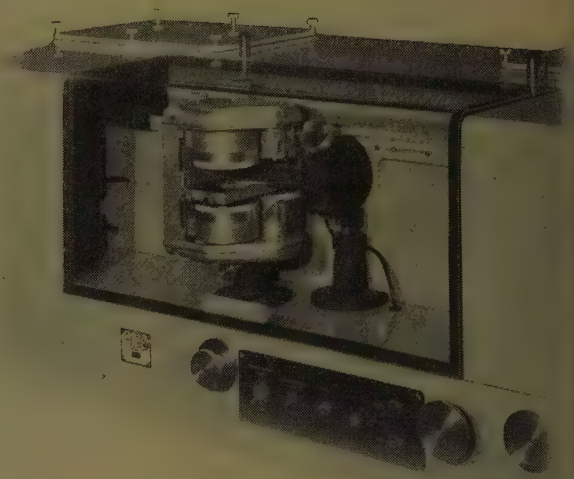
infraröd spektrofotometer

kan nu leveras med en *dubbelstrålig* mikrotillsats för undersökning av mycket små substansmängder.



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BERGMAN & BEVING AB
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Laboratorieutstyr:

Elliott volumetriske glassvarer

Pyrex laboratorieglass

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Bie & Berntsen van Slyke apparat

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– og alt laboratoriemateriell forøvrig.

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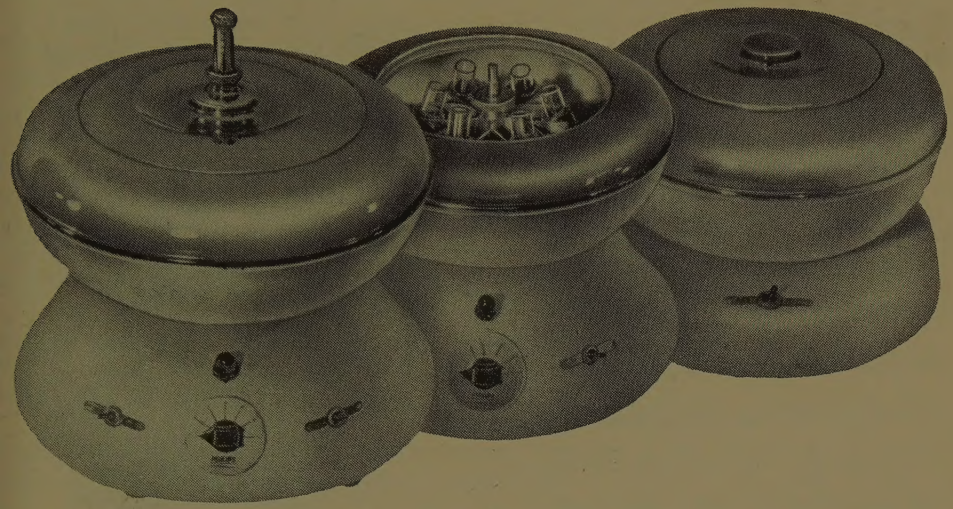

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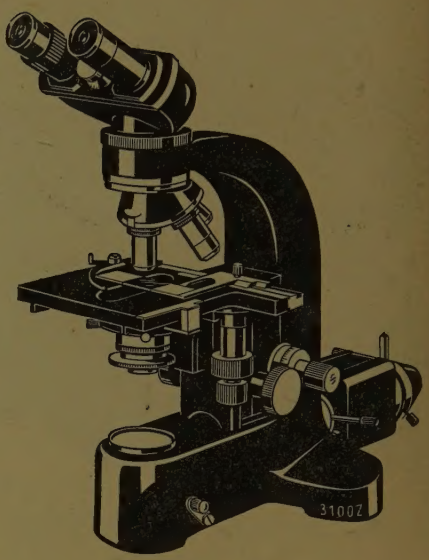
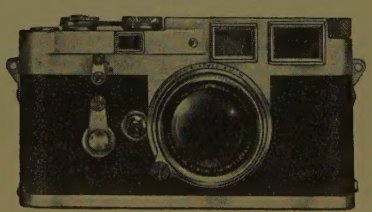
I hela världen är Leitz' optiska forsknings-instrument kända såsom sär klassiga hjälpmedel för forskaren i dennes arbete.



Leitz Dialux

Pääedustaja Suomessa v. 1891 lähtien:
Generalagent för Finland sedan 1891:

Leitz



OY. Christian Nissen A.B.

HELSINKI HELSINGFORS
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